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Functional and Phenotypical Analysis of the Effects of Aging on B Cells and Their Bone Marrow Microenvironment

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LOYOLA UNIVERSITY CHICAGO

**FUNCTIONAL AND PHENOTYPICAL ANALYSIS OF THE EFFECTS OF
AGING ON B CELLS AND THEIR BONE MARROW MICROENVIRONMENT**

**A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN THE CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE**

PROGRAM OF MICROBIOLOGY AND IMMUNOLOGY

**BY
NICOLE MAY ZIEGLER, B.S.**

CHICAGO, ILLINOIS

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
°C	degrees centigrade
acLDL	acetylated low density lipoprotein
APRIL	a proliferation inducing ligand
BrdU	5'-bromo- 2- deoxyuridine
CD	cluster of differentiation antigen
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
Ig	immunoglobulin
IL	interleukin
LPS	lipopolisaccharide
LTBMC-B	long-term bone marrow culture for B lymphocytes
min	minute
ml	milliliter

mRNA	messenger RNA
ng	nanogram
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
RAG	recombination activation gene
RNA	ribonucleic acid
SA	streptavidin
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TLR	toll-like receptor
TNF	tumor necrosis factor

ABSTRACT

Aging impacts multiple organ systems, and specifically causes the immune system to lose its ability to efficiently fight off infections (23, 30, 53, 70). Regarding immunity, aging research predominantly focuses on the adaptive immune system. Experiments illustrate the involution of the thymus as the host ages, which consequently lead to the decline of newly developed T cells. Additionally, thymic involution negatively affects the overall function of T cells. B cells, which mediate the humoral arm of the adaptive immune system, develop throughout life in the bone marrow where microenvironmental ‘niches’ are important. The bone marrow does not exactly ‘atrophy’ with age; however, studies comparing young and old mice demonstrate an age-related change in the bone marrow B cell subpopulations. Changes in the aged bone marrow reflect an increase in mature B cells and plasma cells, possibly due to an increase in available niches, since there is a decline in immature B cells which also reside in these niches (22, 23, 41, 49-51, 53, 70, 76, 77, 80, 82). Despite increases in antibody producing plasma cells, there is an age-related decline of high affinity antibodies, dampening the overall humoral response in the aged host (30, 49, 53). The decline in high affinity antibodies is common in the elderly, providing an avenue in expanding our knowledge of the bone marrow, and its effects on the humoral immune system.

Once humans and other species reach adolescence, the marrow of long bones (for example, femurs) converts from red to yellow marrow (4, 17). Marrow conversion alters the production of multiple blood lineages, including lymphocytes. However, bone marrow from other sites do not undergo conversion, suggesting there are different hematopoietic marrows (45). An example of a non-converting bone marrow is located in the sternum. Structurally, the femur and sternum are different. The femur is cortical bone; the sternum is membranous bone. With notable differences in bone marrow conversion and structure, the femur and sternum may exhibit different cellular composition in their bone marrow microenvironments. For example, bone marrow from femur versus sternum may contain different resident lymphocytes. The difference in bone marrow structure and marrow composition led me to hypothesize that the femur and sternum represent different bone marrow microenvironments in regard to plasma cell frequency and ability to support plasma cell survival *in vitro*. In particular, I hypothesize that there will be an increased frequency of plasma cells in the sternum, compared to the femur, since the sternum remains a site of hematopoiesis for the duration of the host's life. The overall goal of Aim 1 and part of Aim 2 was to determine if femoral and sternum bone marrow have different plasma cell composition and growth factor production that may affect antibody production and secretion. The goal of the first aim was to determine if bone marrow from different sites represent similar or different plasma cell niches. To compare the femur and sternum, I assessed murine bone marrow because

plasma cells accumulate there with age (91). Although mice do not undergo the red to yellow bone marrow conversion, I questioned whether the bone marrows from these two sites are different in regard to plasma cell frequency and survival. In order to answer this question, I first assessed plasma cell frequency by immunofluorescence for intracellular immunoglobulin and subsequently assessed plasma cell survival by co-culturing with bone marrow-derived stromal cells from either bone marrow source.

Plasma cells require various signals from the microenvironment for their maintenance and survival *in vivo*. One particular signal is provided from the TNF family ligand, APRIL (A Proliferation Inducing Ligand) (5, 7). TACI (Transmembrane Activator and Calcium Modulator and Cyclophilin Ligand Interactor), a receptor for APRIL, is found on all peripheral B cells (89). The TACI and APRIL interaction has been shown to be important in maintaining plasma cell number and function in both the spleen and bone marrow. For example, mice treated with neutralizing TACI-Ig demonstrated a decrease in both the frequency of mature B cells and overall antibody production (29, 68). However, current research is lacking on the expression of both TACI and APRIL in aged mice. The overall goal of my second aim was to determine if the expression of APRIL and its receptor TACI are altered in aged mice. Since there is an accumulation of mature B cells and plasma cells in aged bone marrow, I predicted an

increased expression of APRIL mRNA in the both bone marrow sites and TACI on femoral bone marrow B cells of aged mice, compared to young.

This study sought to determine the effects that age and cellular origin have on the bone marrow microenvironment. The conclusions drawn from this work will help expand the known mechanisms required for an efficient humoral response in the host.

CHAPTER I

LITERATURE REVIEW

Humoral Immunity and B Lymphocyte Maturation

We are constantly exposed to harmful agents, yet we remain relatively healthy. Protection is governed in part by the humoral immune system. Humoral immunity is mediated by plasma cells and their production of antigen-specific antibodies. These proteins are secreted into the blood and lymphatics to target and eliminate foreign antigens that could otherwise be detrimental to the host (1, 36, 78). Protection is long-lasting, and antigen-specific antibodies are detected in the serum of humans for decades (78). Additionally, the humoral immune response generates non-immunoglobulin secreting memory cells to the antigen that confer a quick and robust response upon subsequent encounters with the antigen.

The development of the humoral immune system begins in the bone marrow from hematopoietic stem cells (HSCs) (81) (Figure 1). HSCs have the potential to differentiate into multiple lineage specific progenitors, including the common lymphoid progenitor (CLP) (48). CLPs undergo further differentiation to become pre-pro B and pro-B cells, which is dependent on cell contact and additional factors provided by the microenvironment (26, 32, 35). During this process, pro-B cells undergo immunoglobulin heavy chain VDJ gene rearrangement. Upon successful rearrangement,

these now pre-B cells express the pre-B cell receptor and begin rearranging their light chain. Immunoglobulin gene rearrangements are essential for the generation of a diverse antibody repertoire. After functional pairing of the heavy and light chains, immature B cells express IgM on their surface. Immature B cells will leave the bone marrow and migrate to the spleen to continue their maturation before re-circulating to the secondary lymphoid tissues as mature B cells ready for antigen encounter. Mature B cells may encounter their specific antigen and become activated through interactions with an antigen-specific T cell (31). At this stage, an activated B cell can become either a memory B cell or a plasmablast, which is the precursor for antibody producing plasma cells (1). Typically, maturation and differentiation occurs when an activated B cell enters a germinal center reaction and undergoes affinity maturation and isotype switching (52) (Figure 1).

After antigen encounter, some antigen-specific plasmablasts migrate from the spleen to the bone marrow, where they differentiate into long-lived plasma cells (15, 69, 79) (Figure 2). Appropriate migration into the bone marrow requires the interaction between the chemokine CXCL12 and its cognate receptor CXCR4 that is found on plasmablasts (33, 67). CXCL12 is produced by the bone marrow microenvironment, which is thought to generate a chemokine gradient that directs plasmablasts to the bone marrow. Once plasmablasts migrate to the bone marrow, they lose their ability to

respond to CXCL12 (34). However, CXCL12 regulated chemotaxis is sufficient to direct plasmablasts to their appropriate bone marrow niches where they differentiate into plasma cells (see second half of Figure 2). When they have properly migrated and are appropriately situated, newly generated plasma cells produce hundreds to thousands of immunoglobulin molecules per minute (12, 88).

Long-Lived Plasma Cells

The half-life of an antibody is approximately three weeks (88). This suggests that the specific antibody production needs to be continuously replenished by plasma cells. Since serum concentration of antigen-specific antibodies remains constant for years after exposure, the source of antibodies has been controversial (3, 78). The controversy has caused scientists to be in one of two camps of thought in regard to the source of these protective antibodies. The first group believes that the long-term antibody protection is governed by long-lived plasma cells. The second group hypothesizes that plasma cells are not long-lived, but rather continuously generated by memory B cells.

In order to provide adequate protection, the body generates antigen specific plasma cells upon antigen exposure. Proliferation assays, utilizing thymidine incorporation, demonstrated that the plasma cells in the secondary lymphoid tissues were different from those in the bone marrow (37). It appears that two distinct plasma cell populations are generated after antigen encounter (37). The first population consists of

the short-lived plasma cells (37, 39, 40). These cells are formed in the secondary lymphoid tissue within a week of antigen encounter. They provide a primary rapid response. However, their antibodies have low affinity for the antigen, so additional protection is likely needed (39, 40). Additional immunity and protection is facilitated by the long-lived plasma cells, which reside predominately in the bone marrow (6, 20, 59, 79). Long-lived plasma cells are generated in germinal center reactions in the secondary lymphoid tissue and are independent from the memory B cells that are also generated in this response (18). Germinal center-derived plasma cells require more time to mature compared to short-lived plasma cells, and they produce antibodies with high affinity.

Although distinct populations of plasma cells were discovered in the mouse, the lifespan of long-term plasma cells was not determined until the mid 1990s (59, 79). Bromodeoxyuridine (BrdU) incorporates into newly synthesized DNA and is used to detect proliferating cells (59). BrdU experiments allowed the researchers to label cells generated immediately after immunization. Plasma cells generated during the immunization would be labeled since plasma cells are terminally differentiated and do not proliferate, while pre-existing plasma cells generated by prior antigen exposure would not be labeled. The BrdU approach determined that long-lived plasma cells remain in the bone marrow of mice for hundreds of days after immunization, with a lifespan similar to memory B cells. The lifespan similarity led to the speculation that long-lived plasma

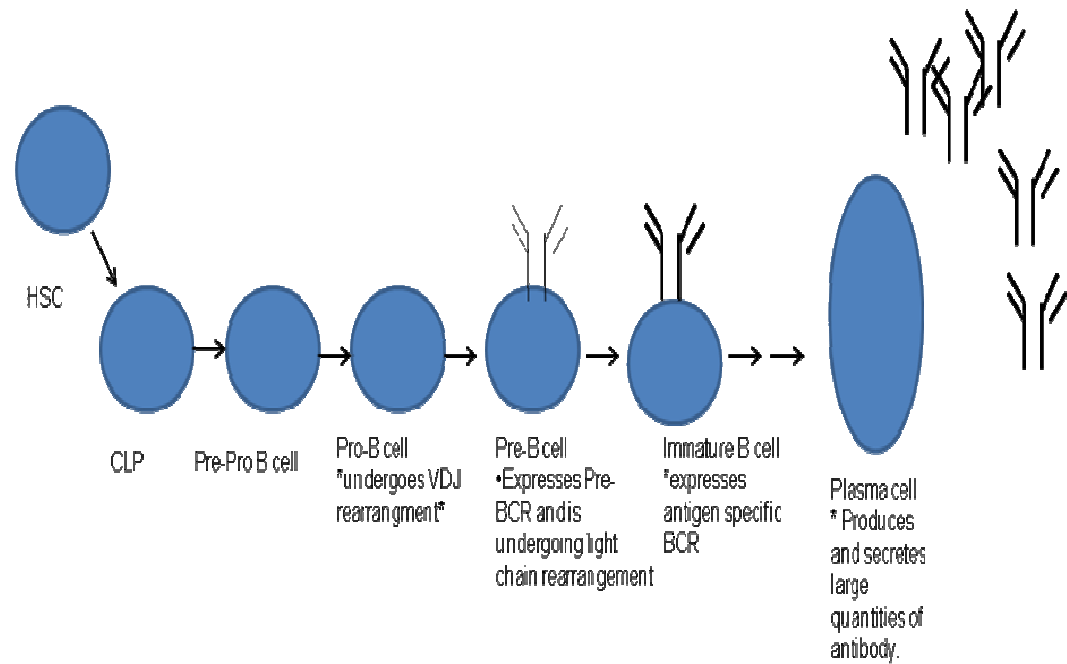


Figure 1: B cell development in the bone marrow. The bone marrow is the site of B cell development. Hematopoietic stem cells (HSC) are generated in the bone marrow and can give rise to multiple cell types, including the common lymphoid progenitor (CLP). A CLP can become a B cell, and B cells require multiple stages of maturation, to become an antigen-specific immature B cell.

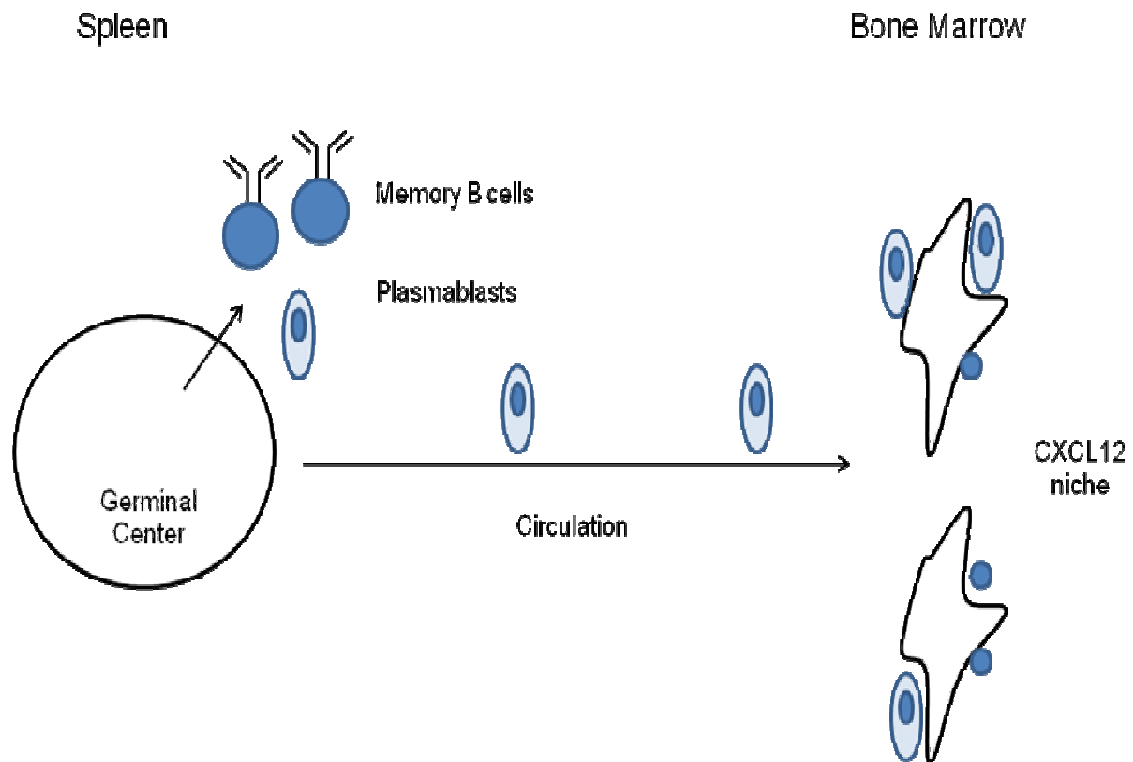


Figure 2: The generation, migration, and maintenance of plasmablasts.

Upon encountering their cognate antigen, B cells undergo a germinal center reaction, leading to the production of antigen-specific memory B cells and plasmablasts. Plasmablasts can then enter the circulation and migrate to the bone marrow, due to their attraction to the chemokine CXCL12. Once situated in the niche, plasmablasts mature into terminally differentiated plasma cells and produce antibodies.

cells are actually maintained by memory B cells in the bone marrow. To determine what role, if any, memory B cells play in humoral immunity, mice were treated with CD20 mAb (18). CD20 mAb depletes mature and memory B cells, while leaving plasma cells intact. If memory B cells contributed to the survival and maintenance of long-lived plasma cells, then a decrease was expected in immunoglobulin production upon CD20 mAb treatment. However, depletion of memory B cells did not decrease the production of serum antibodies, demonstrating that antibody titers are maintained by long-lived plasma cells, not memory B cells.

Bone Marrow Microenvironmental Niches for Plasma Cells

Many cell types, including committed B-lineage cells, require specific niches provided by the microenvironment that offer cell-cell contact and other signals important for maintenance and survival. In particular, plasma cells require extrinsic survival signals for their long-term maintenance (73). *In vitro* co-culturing experiments demonstrated that plasma cells are unable to survive in the absence of additional bone marrow cells (11, 62). Research suggests that the bone marrow reticular stromal cell may provide this plasma niche. The reticular stromal cell is a form of connective tissue cell that have highly branched (dendritic) processes *in vivo* and fibroblastic morphology *in vitro*. In secondary lymphoid tissues, like the lymph nodes and spleen, stromal cells make contact with lymphocytes and generate an extracellular- matrix to provide additional structural

support (42, 71). This contact is also suggested in the primary lymphoid tissue as well (71). The evidence that reticular stromal cells may provide the plasma cell niche is partly due to their ability to produce copious amounts of CXCL12 (9). The engagement of CXCR4 on plasma cells, with its ligand CXCL12 has a positive effect on plasma cells *in vivo*. For when this interaction is inhibited, such as in experiments deleting CXCR4, there is a decrease in the number of mature plasma cells found in the bone marrow (84). This suggests that CXCL12 is required for plasma cell homeostasis in the bone marrow (84). These receptor depletion experiments may recapitulate what occurs *in vivo*. During inflammation at a secondary site, the production of CXCL12 is decreased (86). This decline in CXCL12 causes plasma cell numbers to decline in the bone marrow, and increase at the inflamed sites in the host (57). Additionally, Tokoyoda's work supported the hypothesis of reticular stromal cells providing a plasma cell niche by demonstrating that the majority of IgG⁺/CD138⁺ plasma cells were in direct contact with CXCL12⁺ stromal cells (84). In addition to providing niches for plasma cells, CXCL12⁺ stromal cells also support early B cell lymphopoiesis (44). Overall, these experiments suggests that bone marrow-derived stromal cells likely provide *in vivo* plasma cells niches, and would be an ideal candidate for use in *in vitro* culturing systems.

Besides reticular stromal cells, dendritic cells may also comprise a plasma cell niche in the bone marrow (27, 75, 83). Experiments assessing splenic CD11c⁺ dendritic

cells demonstrated that they are associated with newly generated plasmablasts (27). Phenotypically similar dendritic cells have been characterized in the bone marrow (75). Elegant experiments eliminating the bone marrow-derived dendritic cells lead to the prevention of antigen-specific IgM producing plasma cells *in vivo* (75). This outcome demonstrates that dendritic cells contribute to plasma cell maintenance in the bone marrow.

Although two potential microenvironmental niches (reticular stromal cells and dendritic cells) have been elucidated, many questions remain unanswered in regard to the extrinsic signals and niches that are required for plasma cells *in vivo*. First, do reticular stromal cells and dendritic cells represent the only two elements of the plasma cell niche, or do other cell types play a role? Second, since plasma cells accumulate with age, are the niches limitless or are they confined to a finite number in the bone marrow? Third, since antigen-specific antibodies are found in the circulation years after antigen exposure, do plasma cells compete to remain in their survival niche or is their residence there permanent? Lastly, are all bone marrow microenvironments equivalent or distinct based on its location in the animal?

Changes in the Bone Marrow and B Cell Repertoire With Age

As we age, many organ systems begin to decline. Much scientific effort has focused on understanding how aging impacts the immune system. In regard to the

humoral immune response, aging negatively impacts the antibody response, leading to a decline in the antibody diversity that limits the overall effectiveness of the response (30, 53, 80). In addition to intrinsic alterations in the production of immunoglobulin, changes in the microenvironments that support the humoral part of the immune system, occur in the older host (30, 50, 82).

One noticeable change in the murine bone marrow is that the residing B cell population deviates with age (41) (Figure 3). Studies characterizing the percentage of cells at each developmental stage in the bone marrow demonstrate an age-related shift from an immature to a mature phenotype (41). Even though the aged bone marrow microenvironment favors an increase in mature B cells and plasma cells, the antibody repertoire is less diverse compared to younger mice (53). This is due to the reduced somatic hypermutation during the VDJ rearrangement of the immunoglobulin heavy chains generated in aged antibody producing cells (plasmablasts and plasma cells) in both the bone marrow and the spleen (53). Similarly, antibodies in aged human subjects have lower avidity and affinity for their specific antigen than those from younger individuals (46, 47). Lower avidity and affinity negatively affects the interactions of antibodies to their cognate antigen, which in turn decreases their protective capabilities. In some cases, the decline in antibody diversity is associated with a decrease in total serum antibodies (30, 47).

A decrease in circulating antibodies contributes to inefficient responses against invading pathogens. Various groups have observed that plasma cells accumulate in the aged bone marrow (91). However, work assessing newly generated antigen specific antibody forming cells (AFCs), which contain both plasmablasts and plasma cells, demonstrated a decrease in overall generation of AFCs and AFCs in the aged bone marrow after immunization (30). These data suggest that the evident plasma cell accumulation may be comprised more of plasma cells generated earlier in life. Since follicular B cell maturation depends on the help provided from CD4⁺ T cells, research has focused on determining whether the decline in the humoral response with age is due to a deficiency in CD4⁺ helper T cell function. Song et al. demonstrated that there was a decrease in CD4⁺ helper T cell function with age (80).

Investigators have addressed changes in the secondary lymphoid tissue, in particular, the germinal center events that occur there. The secondary lymphoid tissues, such as spleen, are sites of B cell maturation and activation due to their exposure to antigens and formation and completion of germinal center reactions. In the aged mouse model, there is a decline in the generation of plasmablasts and plasma cells after immunization (30, 31). A potential explanation for the decrease in the production of AFCs after immunization is that germinal center reactions decrease with age. Assessment

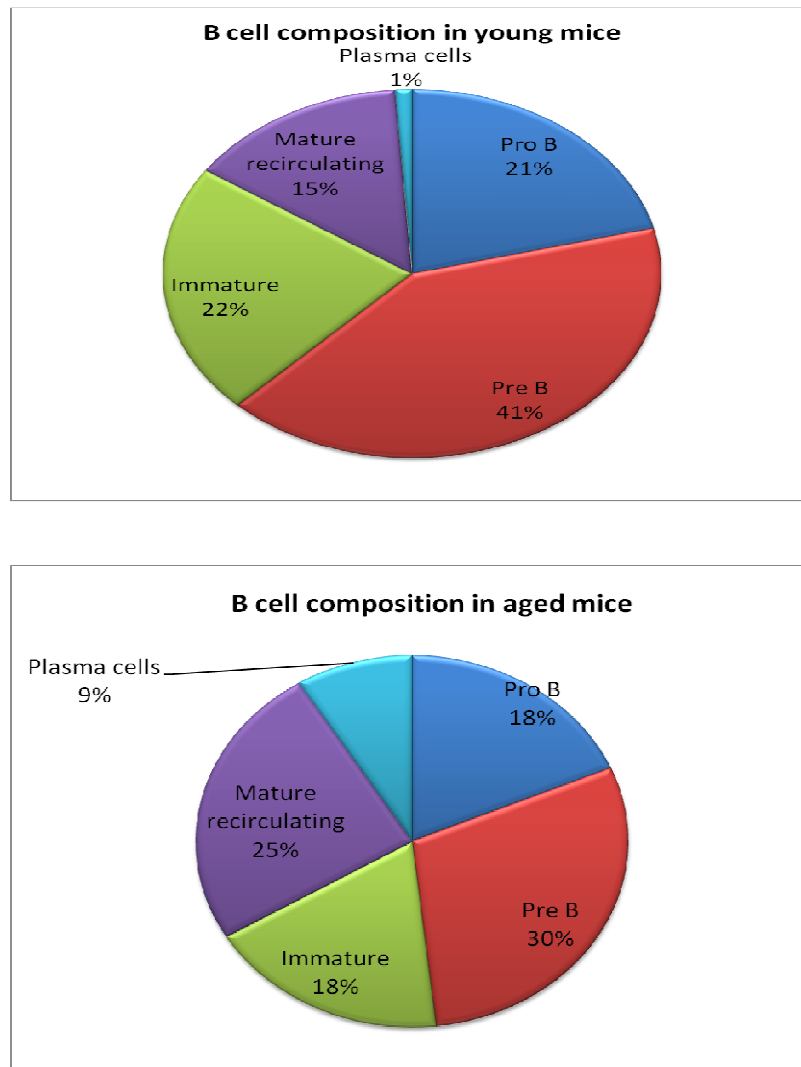


Figure 3: Percentage of B-cell subsets within the total bone marrow B-cell population. Bone marrow was flushed from young (2 month old, N=56) and old (22 month old, N=51) BALB/c mice and were stained with various antibodies. B cell phenotypes were assessed and quantified by flow cytometry analysis. pro-B, B220lo/CD43+/IgM±; pre-B, B220lo/CD43±/IgM±; immature B, B220lo/CD43±/IgM+; mature recirculating, B220hi; plasma cells, B220neg/CD138+. Data courtesy of Kara Johnson, PhD (41).

of splenic sections after immunizations demonstrated that there is a decline in both the formation of germinal centers and the number of germinal center B cells, suggesting that aged spleen cannot properly facilitate this response (30).

The impact aging has on the immune system is likely affected by changes in the bone marrow itself. After immunization, the bone marrow becomes the primary site of antibody production. So, changes in the marrow microenvironment could be detrimental to this arm of the humoral immune response. In addition to changes to the plasma cell compartment, age related changes in the bone marrow microenvironment affect B-lymphopoiesis. Adequate antibody responses require efficient immunoglobulin gene rearrangement, which is mediated by RAG 2, the enzyme important in rearrangement and recombination of the immunoglobulin and T cell receptor. Bone marrow chimeras determined that the decreased RAG 2 and recombinase expression and activity was controlled by extrinsic factors contributed from the bone marrow itself (49, 50). Work done in our lab determined that there is a decrease in early B cells in aged long-term bone marrow cultures (LTBMC) (82). This decrease was caused by insufficient IL-7 produced from the bone marrow stromal cells, which is required for the survival of early B cells. Additional work using LTBMCs demonstrated that these aged stromal cells also generate increased concentrations of CXCL12 (Su et al. in preparation). Changes in the production of some bone marrow derived growth factors may represent a shift in the

types of niches present in the aged bone marrow. The alteration of extrinsic signals and growth factors may explain why there is an accumulation of long-lived plasma cells in the bone marrow of aged mice, seemingly at the expense of early B cell subsets (Figure 3).

Femur versus Sternum

Vertebrates rely on their skeleton to provide structural support and protection of their organs. Additionally, bones can harbor the bone marrow, which is the major site of hematopoiesis (45). The skeleton is comprised of structurally different bones, based on their development and overall function. Cortical bones, such as the femur, support the body with its strong and durable outer shell (cortex). Cortical bones develop from the growth plates by cartilage growth and the overall calcification of the cartilage. Encased by the cortical bone of the femur is the bone marrow. At birth, the red bone marrow is the site of hematopoiesis, which produces red blood cells, platelets, and white blood cells (45). By the time a human reaches adolescence, the human femoral bone marrow converts from red marrow to yellow marrow, which is comprised of adipocytes (17, 25). This reduction in hematopoiesis is due to the conversion of the marrow as well as the decline in hematopoietic stem cell (HSC) function (87).

In contrast to the femur, the sternum is a long membraneous flat bone. Membraneous bones typically develop from clusters of mesenchyme, rather than cartilage. Mesenchyme is an example of undifferentiated reticular connective tissue,

which is a form of loose connective tissue. The sternum protects the chest from trauma and houses red marrow. However, there are two notable differences between the bone marrow of the sternum and that of the femur. The sternum does not undergo conversion from red to yellow marrow, and remains a site of hematopoiesis for the duration of life. Additionally, the sternum in the mouse is formed in segmented joined with small cartilage plates, while the femur is continuous (Figure 4). This discontinuation of marrow may alter the B cell niches that reside in the sternum bone marrow. Despite these differences in the compartmentalization of bone marrow, Trubowitz et al. demonstrated that the sternum harbors similar vascular circulation to the femur, demonstrating that circulation is not a factor in the potential difference in niches (85). This result suggests that regardless of the differences in the structure and composition of these two sites, hematopoietic cells are able to migrate to and from the bone marrow in a similar manner.

Since these two bone marrow sites differ in regard to the balance of red marrow and compartmentalization, it suggests that the total body bone marrow in adults is not uniformly distributed. Research in Dr. Phong Le's lab has demonstrated that there is an increased expression of the transcription factor Foxn1 in the sternum compared to the femur in Foxn1 transgenic mice (Zook *et al.* in preparation). Mutations in this

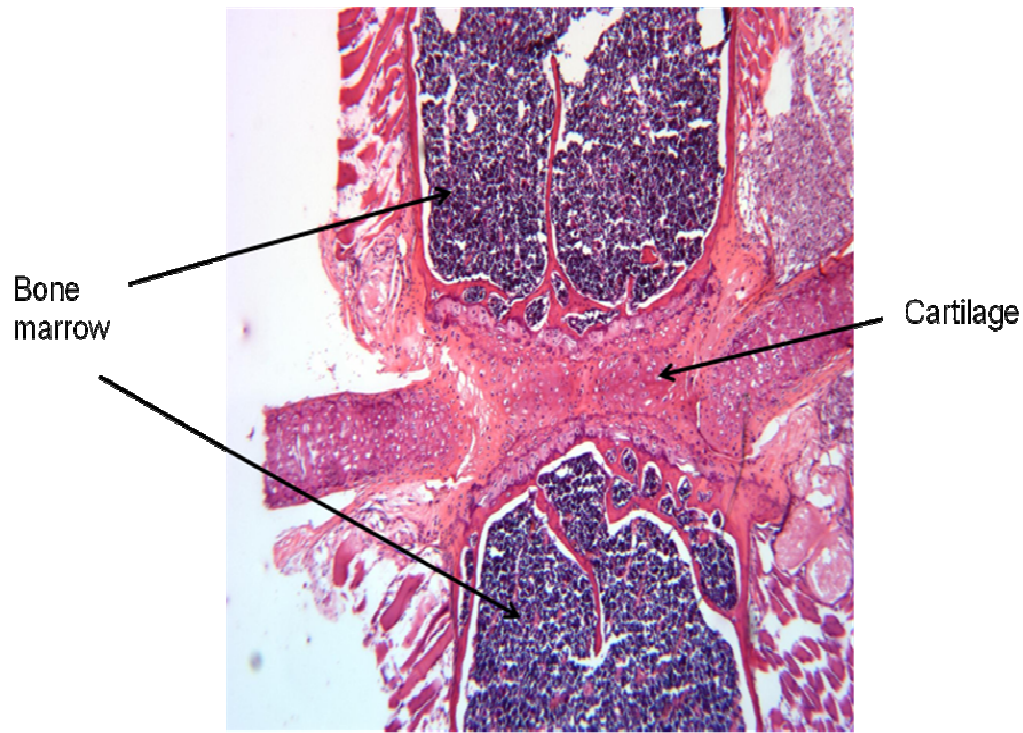


Figure 4: The organization of the sternum bone marrow.

Hematoxylin and eosin staining was used to illustrate the segmentation of the sternum bone marrow. The bone marrow (dark purple) is segmented by cartilage (light pink) in this 24 month wild-type C57Bl/6 mouse. Sternum sectioning and staining performed by Erin Zook and Shubin Zhang. (6.3x magnification)

transcription factor correlate with deficiencies in the T cell compartment of the immune system. This increased expression of Foxn1 may explain why the sternum has an increased frequency of early lymphoid progenitors, like the common lymphoid progenitor (CLP), in these mice (Zook *et al.* in preparation). Though Zook's current research has initiated the exploration of the differences in bone marrow cellular populations, my document is one of the first to look at the sternum and its resident B cell compartment.

Plasma Cell Signals for Survival or Immunoglobulin Regulation

An efficient antibody memory depends on the ability of plasma cells to survive for long periods of time in the bone marrow. Plasma cell survival is governed by soluble factors and cell contact provided by the local microenvironment (2, 5, 7, 10, 11, 15, 18, 21, 24, 27, 28, 30, 37, 38, 43, 58-60, 62-64, 68). This is predominately provided by the reticular stromal cells (10, 11, 42, 62). However, the heterogeneity of the bone marrow suggests contributions from additional cell types. A summary of the important plasma cell signals for this thesis are listed in Table 1.

The factors secreted by reticular stromal cells have been studied to determine their contribution to plasma cell homeostasis. One well known factor is the chemokine CXCL12. Upon engagement of CXCL12 to its receptor CXCR4, plasmablasts and plasma cells migrate towards the bone marrow, which has an increased concentration of

Receptor/Surface Molecule	Type	Function	References
CD138	Transmembrane heparan sulfate proteoglycan	Cell signaling, and can bind to APRIL	Ingold et al. 2005
CXCR4	Chemokine receptor for CXCL12	Important in proper homing to bone marrow, and other CXCL12 rich areas	Hauser et al. 2002, Minges Wols 2007, Cenci et al. 2007
TACI	Transmembrane protein that binds to TNF family ligands	Important in cellular activities like antibody production, plasma cell survival, and homeostasis	Chu et al. 2007, Darce et al. 2007, Xu et al, 2001, Mackay et al. 2007
TLRs	Single membrane spanning receptor	Antibody production due to these signals from foreign antigens	Dorner et al. 2009, Genestier et al. 2007, Ingold et al. 2005

Table 1: Receptors and other plasma cell surface molecules important in plasma cell signaling.

this chemokine, compared to the secondary lymphoid tissues (34, 60). Though CXCL12 controls the accumulation of plasma cells to the bone marrow, it does not appear to modify immunoglobulin production by itself (60, 67). Though isolated plasma cells cultured with CXCL12 produce and secrete antibodies, greater concentrations are generated when plasma cells are incubated with another stromal cell-derived factor, interleukin-6 (IL-6) (60). IL-6 is produced by multiple types of cells, and is found in higher concentrations in the secondary lymphoid tissue compared to the bone marrow (60). Though IL-6 is not as highly expressed in the bone marrow, IL-6 is required for the survival of plasma cells *in vitro* (11). However, IL-6 knock-out mice had similar numbers of plasma cells compared to wild type (11). This suggests that IL-6 is sufficient but not the only factor required for plasma cell homeostasis.

Recently, APRIL was discovered as a factor important in bone marrow plasma cell function and maintenance. This tumor necrosis factor (TNF)-family member is expressed in both the membrane-bound and soluble form and is produced primarily by innate immune cells, like monocytes (56). APRIL is typically found as a homotrimeric transmembrane protein, which is proteolytically cleaved, and upon multimerizing with additional APRIL proteins, it can engage its cognate receptors. APRIL can bind to both the BCMA (B Cell Maturation Antigen) and TACI receptors that are expressed on various B cell types (54). TACI is expressed on all peripheral B cells, including plasma

cells, while BCMA is restricted to plasma cells alone (13, 16). In addition to APRIL, BCMA can also be activated when it binds BAFF (B cell Activating Factor) (54). APRIL can also bind to heparin sulphate proteoglycans (HSPG) like syndecans, which are expressed on plasma cells (38).

Studies utilizing BCMA knock-out mice demonstrated that BCMA is important for long-term plasma cell survival (68). The researchers demonstrated that BCMA knock-out mice had a significant decrease in the number of antigen-specific ASCs in the bone marrow, for seven weeks after immunization. Our lab, and others, determined that transcripts for one of BCMA's ligands, BAFF, are expressed in very low amounts in the total bone marrow or specifically, the stromal cells (5, 60). This finding suggested that BCMA's other ligand, APRIL, may be an important signal for *in vivo* plasma cell survival in the bone marrow. Additional experiments determined that in the absence of endogenous APRIL, there was a decrease in the number of plasmablasts and plasma cells in both the primary and secondary lymphoid tissue (5, 7). APRIL can also affect antibody diversity by regulating class switching (5). APRIL knock-out mice had decreases in isotype class switching, in particular, switching to the IgA isotype (5).

Once it was determined that APRIL-dependent signals positively regulated plasma cell survival, researchers next wanted to determine which receptor, BCMA or TACI, mediated the plasma cell survival. Conflicting reports have emerged in regard to

BCMA's role in APRIL-mediated signaling. Xu *et al.* found that in BCMA knock-out mice, there was no negative effect on immunoglobulin production or germinal center formation compared to wild-type mice (90). However, later studies illustrated a significant decline in antigen specific long-lived plasma cells in the bone marrow (68). These data suggest that BCMA does not play a prominent role in the formation of plasma cells, or their function, but rather their survival in the bone marrow. Research regarding TACI's role in APRIL-mediated responses, has been more straight-forward. In mice deficient in TACI, there is an increase in B cell proliferation, indicating that TACI regulates B cell homeostasis (55). Specifically, the lack of TACI lead to a significant decrease in IgA class switching, demonstrating that the previous observations about APRIL's regulation were mediated by the TACI receptor on plasma cells (55, 74).

Cell regulation is complex and causes multiple factors to contribute to similar cellular functions. Another signaling cascade that regulates plasma cell function is provided by toll-like receptors (TLR). These pattern recognition receptors (PRR) recognize molecular patterns commonly found on pathogens and were originally identified on cells of the innate immune system (21). Studies assessing TLR mRNA levels in various B cell populations determined that all B cells express some TLR transcripts; however, the profiles varied depending on the particular B cell subsets (21). Both human peripheral blood and tonsil residing plasma cells express TLR1-TLR9 (8,

21). Engagement of these TLRs led to an increase in both antibody production and secretion (21, 28, 43). In mouse, TLR agonists signal for the differentiation of various B cell subsets to differentiate into plasma cells (28). Similar to human plasma cells, TLR signaling leads to an increase in immunoglobulin secretion and expression of plasma cell transcription factors (like Blimp-1) in B cells. This response occurs in the secondary lymphoid tissue, which could lead to the generation of long-lived plasma cells in the murine bone marrow. The signals could be due to TLR signaling itself, or to subsequential signaling after TLR engagement. When terminally differentiated plasma cells were exposed to TLR7 and TLR9 agonists, there was an up-regulation of the TACI receptor (43). These results demonstrated that TLR signaling not only affects the innate immune response, which is non-specific, but also contributes to the adaptive immune response.

Summary

The generation and survival of plasma cells is essential for providing an immediate and long lasting humoral protection to foreign antigens. Upon activation, antigen-specific B cells differentiate into plasmablasts and migrate to the bone marrow. There, they further differentiate into plasma cells in response to extrinsic factors contributed by the bone marrow microenvironment. These plasma cells reside in niches, comprised of stromal cells and dendritic cells, and provide protection to the host by producing high concentrations of antibodies.

The bone marrow represents a dynamic microenvironment, one which can change its cellular composition and production of extrinsic factors. Marrow is housed in both cortical and membranous bones. Cortical marrow changes as the host ages. Although plasma cells accumulate in bone marrow throughout a lifetime, the ability of new plasma cells to adequately protect diminishes, due to their production of low affinity and less diverse antibodies. Understanding the contribution the bone marrow provides for this response and how it may change as the host ages is important in modifying vaccines to stimulate efficient responses in the elderly.

The purpose of my thesis is to investigate two bone marrow sites and determine their similarities and differences in regard to plasma cell survival and immunoglobulin production. This thesis will look at the impact bone marrow location and age have on the

plasma cell frequency and *in vitro* survival. Overall, my experiments will help determine if the previous research on femur-derived plasma cells represents a universal paradigm or applies only to the plasma cells residing in the femur. This knowledge will help aid in the quest to understand the mechanisms and factors required for an adequate humoral attack in response to a foreign antigen.

CHAPTER II

METHODS

Mice

Young (6 week) and old (18 months) female BALB/c mice were purchased from Harlan (Indianapolis, IN) and Charles River/National Institute of Aging (NIA) (Bethesda, MD), respectively. Once received, mice were housed at the Comparative Medicine Facility at Loyola University Medical Center under standard pathogen-free conditions. Mice were sacrificed by CO₂ inhalation. Aged mice with visible tumors or enlarged spleen were not used in these studies.

Antibodies

The following antibodies were used throughout these studies: PerCP rat anti-mouse CD45R/B220 (0.2 mg/ mL; RA3-6B2, BD Biosciences, San Jose, CA), PE rat anti-mouse CD138 (syndecan-1) (0.2 mg/ mL; 281-2, Pharmingen, San Jose, CA), APC conjugated rat anti-mouse TACI (CD267) (0.2 mg/mL; eBio8F10-3, e-Bioscience, San Diego, CA), goat anti-mouse IgG/A/M-FITC (Invitrogen, Carlsbad, CA), affinity chromatography purified rat anti-mouse IgM (11/41, BD Biosciences), affinity chromatography purified mouse IgM isotype control (11E10, e-Bioscience), which was

used to generate a standard curve for an ELISA, and biotin rat anti-mouse IgM (R6-60.2, Pharmingen).

Preparation of femoral and sternum bone marrow

Both tibias and femurs were removed from each mouse. Bone marrow was flushed with a 25-gauge needle attached to a 10 ml syringe filled with long-term bone marrow (LTBMC) media. The media consisted of RPMI 1640 (Lot 15040254, Mediatech, Manassas, VA), supplemented with 5% FBS (Lot ARE26454, HyClone, Logan), 1% L-glutamine (Cellgro, Manassas, VA) 1% penicillin/streptomycin (Cellgro) and 5×10^{-5} M β -Mercaptoethanol (β -ME) (Fisher Scientific, Pittsburgh PA). Total bone marrow cells were quickly vortexed and passed through a syringe and 25-gauge needle to break up any cell clumping. Cells were centrifuged at 1100 rpm for seven minutes and cell pellets were resuspended with 5 ml media and counted by trypan blue exclusion. Typically, two femurs and two tibias from a single mouse yield approximately 45 million live cells, though this number varies with age.

The sternum was removed from each mouse and muscle was removed with forceps. Bone marrow was flushed out with a 25-gauge needle attached to a 10 ml syringe filled with LTBMC media. Since the sternum is segmented, after a section was flushed, the sternum was cut to reach the next marrow section. After flushing, the cells were centrifuged and pellets were resuspended with 1 ml of media and counted by trypan

blue exclusion. Typically, a single sternum yields approximately ten million live cells, although this number varies with age.

Enumeration of plasma cells in cytodots of total bone marrow cells

Bone marrow cells were harvested from wild type young (2-3.5 months) and old (18-24 months) BALB/c mice. Two hundred and fifty thousand bone marrow cells were resuspended in two hundred and fifty microliters of RPMI and cytospun onto each slide (Thermo Scientific Shandon Cytospin3). Cytodots were either stained immediately or dessicated and stored at 4°C. Cytodots were fixed with 95% ethanol/5% acetic acid and stained with the FITC conjugated goat anti-mouse IgM/A/G antibody at a concentration of 0.2 µg/dot and mounted with a coverslip. Approximately 1000 total cells were counted by phase contrast, and plasma cells were assessed and scored by immunofluorescence (Leitz Diaplan Microscope, magnification 20X).

Phenotyping of bone marrow

After the bone marrow was harvested, 1×10^6 bone marrow cells were stained with rat anti-mouse B220-PerCP, rat anti-mouse CD138-PE and rat anti-mouse TACI-APC antibodies, each at a 1:50 dilution (4 µg/mL) in FACS staining buffer. FACS staining buffer consists of Hank's Balanced Salt Solution supplemented with calcium and magnesium, and 5% heat inactivated fetal calf serum and 0.1% sodium azide. Cell suspensions were incubated on ice for 20 minutes. After incubation, cells were washed

with 1 ml of staining buffer. Cells were then centrifuged, at 1100 rpm, and resuspended in 1ml of 1x PBS. After a second centrifugation, cells were fixed with 1% paraformaldehyde in PBS. The cells were stored at 4°C overnight and flow cytometry analysis was performed the following day using the BD FACSCanto flow cytometer (Franklin Lake, NJ) and FlowJo software.

Stromal cell isolation

Whitlock-type long term bone marrow cultures that support B-lymphopoiesis (LTBMC-B) were generated from the bone marrow from either femoral bones and sternum of individual young (age 6-10 weeks) or old (age 15-24 month) BALB/c mice. Femoral cells were plated at a density of 13×10^6 cells/100 mm plate (Corning, Inc., Corning, NY) and grown in LTBMC media. Sternum bone marrow cells were plated at a density of 6×10^6 cells/60 mm plate (Corning, Inc., Corning, NY) and grown in LTBMC media. Although cultured in different sized plates, the adherent growth of cells from femur and sternum appeared similar in culture, and cells from each marrow source reached the desired confluency around week four.

Approximately six weeks following culture set-up, stromal cells were FACS-sorted into individual wells of flat-bottomed 96-well plates (Corning, Inc., Corning, NY) at 1×10^4 cells/well. Prior to sorting, LTBMC-B cultures were incubated for 3.5 hours with 5µg/ml (in a total of 2.5 mL LTBMC-media) 1,1'-dioctadecyl-3,3,3',3'-tetramethylindolocarbo-cyanine perchlorate-labeled acetylated low density lipoprotein

(DiI-ac-LDL) at 37°C (Biomedical Technologies Inc., Stoughton, MA), in order to separate macrophages from stromal cells. After incubation, lymphocytes were removed by the addition of 1x ethylenediamine tetraacetic acid (EDTA), in a total volume of 3 ml. Calcium chelation allows for the disengagement of lymphocytes from stromal cells. Adherent cells were then treated with 0.25% trypsin-EDTA for five minutes and harvested, after neutralization with LTBMC, by gentle scraping with a silicon rubber policeman. Adherent cells were then resuspended in FACS sorting staining buffer, which consists of RPMI 1640, 5% FCS, L-glutamine, gentamycin, and 5×10^{-5} M β -mercaptoethanol. Cells were brought to the FACS facility and were sorted based on high forward and side scatter and the inability to take up DiI-ac-LDL (Figure 5).

Plasma cell isolation

For each isolation, bone marrow was flushed from three individual wild type aged mice, pooled, and counted as previously described. Total bone marrow cells, approximately 130 million cells from three mice, were incubated with rat anti-mouse CD138-PE at a 1:40 dilution (0.005 mg/mL) in staining buffer [Hanks BSS with Ca^{2+} , Mg^{2+} (Fisher Scientific), 5% HI FBS (heat inactivated FBS), and 2mM EDTA]. After a twenty minute incubation on ice, cells were washed in buffer and incubated with anti-PE beads, at 5 μl per million cells (Miltenyi Biotec). After washing, CD138 positive cells were then enriched by column isolation (Miltenyi Biotec MACS Separation Column MS MiniMACS Columns). Column isolation requires an appropriate apparatus: a column

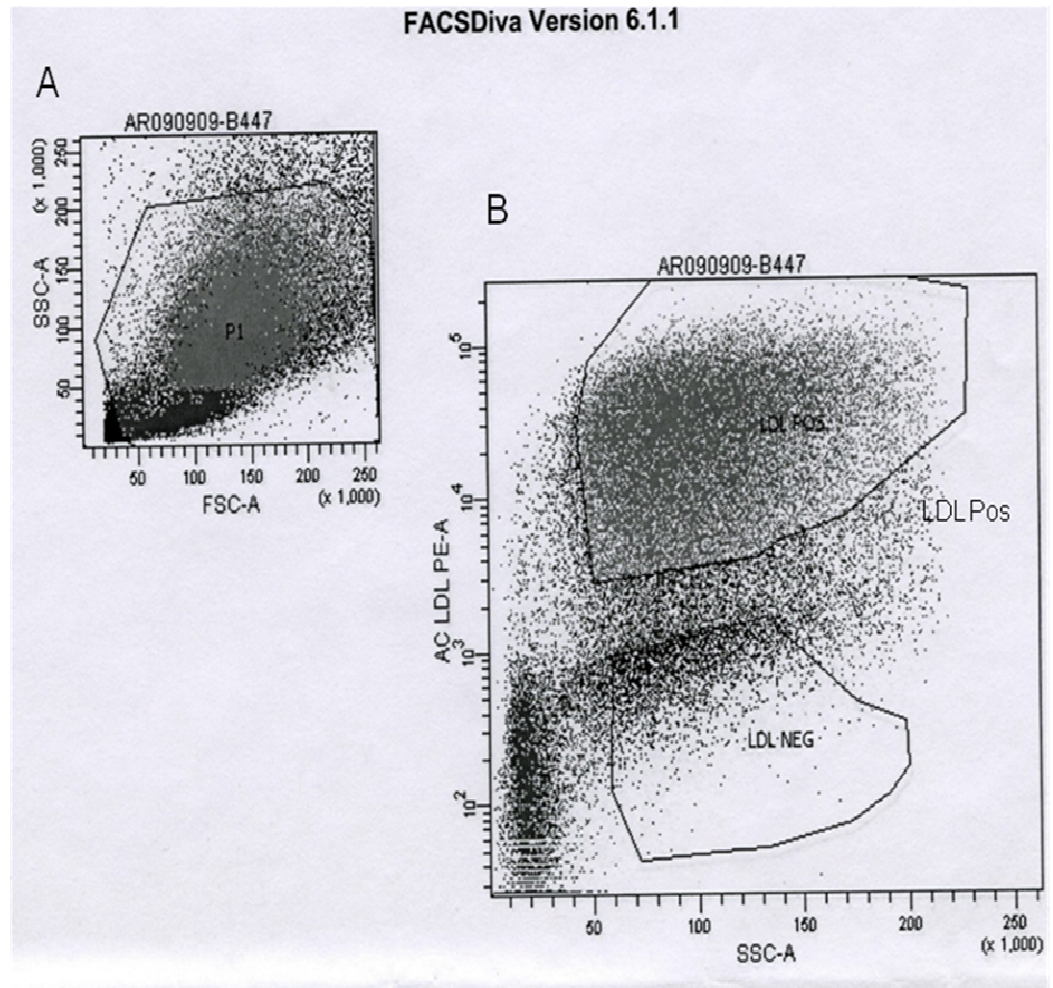


Figure 5: Stromal Cell Profile. After incubation in DiI-ac-LDL, adherent LTBM cells were taken to the FACS facility to be sorted based on their ability to take up ac-LDL and overall size and granularity. Cells are first sorted and gated based on moderate FSC and SSC (A). Gating eliminated any extremely low FSC and SSC cells to reduce the number of dead cells. Desired stromal cells where ac-LDL negative with a moderate granularity (SSC) (B).

attached to a separator, washed with buffer alone. Once the column flow is established, 500 μ l of the cell suspension is added to the column. This is repeated until the entire suspension is passed through the column. To remove residual PE-negative cells, the column was washed two times before the positive cells were plunged into a conical tube. Column-isolated cells were then further purified by fluorescence-activated cell sorting (FACS) for CD138 positive cells. The approximate recovery of CD138 positive cells from 120×10^6 cells was 7.5×10^4 .

Co-culturing of stromal and plasma cells

Isolated plasma cells from wild type bone marrow were added to FACS-sorted stromal cells at a density of 5×10^3 plasma cells per well, with 10^4 stromal cells into 96-well flat-bottom plates. The total volume was then adjusted to 150 μ l. Cells were cultured at 37°C and 5.0% CO₂ for approximately three weeks. Supernatants were collected at various time points and fresh media was added after collection. Supernatants were previously collected at indicated time points and were frozen at -20°C until they were tested by ELISA.

Measurement of immunoglobulin by Enzyme-linked immunosorbent assay (ELISA)

Nunc U-bottom 96 plates (Fischer Scientific, Hanover Park, IL) were coated overnight with 2 μ g/ml rat anti-mouse IgM (BD Bioscience) in 1x PBS with 0.02% sodium azide. Plates were then blocked with 1x PBS/1% BSA at 37°C for one hour to prevent nonspecific binding. To generate a standard curve, each plate contained two-

fold serial dilutions of isotype specific standards, ranging from 1000 ng/ml to 1.95 ng/ml, at a total volume of 40 μ l per well. As a negative control, media alone was used. Once the supernatants were incubated for two hours at 37°C, the detection antibody (biotin conjugated rat anti-mouse IgM) was added to each well at 1 ug/ml for two hours. After washing the plate, streptavidin-conjugated alkaline phosphatase was added for 30 minutes at 37°C. The assay was developed by the addition of p-nitrophenyl phosphate (PNPP) (Sigma-Aldrich) diluted in 1 mg/ml in 10mM diethanolamine, 0.5mM MgCl₂, pH 9.5. Plates were read using a BioTek Synergy HT plate reader (Winooski, VT) at optical density of 405 nm. Results were reported, based on the linear part of the standard curve, in ng/mL.

RNA isolation and Real Time PCR analysis of APRIL

RNA was isolated from either total bone marrow or spleen of young and aged mice. Total bone marrow, from either femur or sternum, was isolated as described above, and cell pellets ranged from 1×10^6 - 10×10^6 cells/pellet. Spleens were cut into fourths and one-fourth was weighed and used for RNA isolation. RNA was isolated from the homogenates using the Qiagen RNeasy kit. Spleens were homogenized using a rotor stator homogenizer, suspended in Buffer RLT (Valencia, CA), which lyses the cells to obtain RNA. After homogenizing cells, 70% ethanol was added to the cells to optimize binding of RNA to the RNA membrane provided in the kit. After cell lysates were added to the membrane, the membrane was washed and centrifuged to eliminate contamination.

After washing, RNA was eluted and the concentration was estimated by NanoDrop technology (Thermo Scientific). RNA degradation was assessed by analyzing RNA gels. cDNA was synthesized using Fermentas RevertAid First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada).

Real-Time PCR analysis was performed using a GeneAmp 7300 Sequence Detection System (Perkin Elmer-Applied Biosystems). SYBR Green PCR Master Mix (Invitrogen, San Diego, CA), forward and reverse primers at 2.5 μ M, UV treated ddH₂O, and appropriate cDNA were added to each sample. As a positive control for APRIL, RNA was isolated from young and old spleens. Negative controls included samples without cDNA. A standard curve for HPRT was generated from plasmids containing their PCR product. These standards underwent ten-fold dilutions ranging from 1×10^1 copies/ μ l- 1×10^5 copies/ μ l. Controls, standards, and samples were run in triplicate in a 96-well optical reaction plate. Primers were designed using Primer Express Software (Applied Biosystems) and Primer3 (SourceForge) and purchased by Invitrogen. Oligonucleotide sequences are shown in Table 2. Primers were generated to span an intron and ends were assessed to prevent primer binding. The Real-Time PCR was run on the following settings: one cycle at 50°C for two minutes, one cycle at 95°C for 10 minutes, 40 cycles of denaturing at 95°C for 15 seconds and annealing and elongation for 60°C for one minute. The data were analyzed using GeneAmp 7300 SDS software (Applied Biosystems). APRIL mRNA expression levels were calculated by determining

the ratio of the number of copies of APRIL to the number of copies of a housekeeping gene (HPRT).

Statistical Analysis

Statistical significance between the experimental groups was determined using unpaired and paired Student's t test using GraphPad Prism 5 software (San Diego, CA).

Amplified Transcript	Oligonucleotide Sequence
HPRT	Forward: AGCAGTACAGCCCCAAAATGG Reverse: TGCGCTCATCTTAGGCTTTGT
APRIL	Forward: CCCATTCCAAAACTGGCTA Reverse: AAAGAATTTGGGGCTGTGTG

Table 2: Oligonucleotide sequence of primers used in Real-Time PCR experiments. Primers were designed using Primer Express Software (Applied Biosystems) and Primer3 (SourceForge), and purchased by Invitrogen.

CHAPTER III

RESULTS- EXAMINATION OF PLASMA CELLS AND THEIR NICHES IN FEMORAL AND STERNUM BONE MARROW

Over the last decade, our laboratory developed an interest in the mechanisms regulating plasma cell longevity in the bone marrow (60, 62, 63). An accumulation of plasma cells is evident in aged bone marrow compared to the number found in young mice. Additionally, long-term bone marrow cultures (LTBMC) generated from aged mice contain resident plasma cells that can survive *in vitro* for several weeks (62). The question of whether the aged microenvironment might favor the survival of plasma cells, both *in vivo* and *in vitro* has not yet been addressed. In regard to plasma cell research, the experimental approach utilized femoral-derived bone marrow. I sought to determine if the same results would be obtained from sternum bone marrow, which is known to develop differently than the femoral bone marrow (17, 45). I compared the frequency of IgM producing plasma cells indirectly via *in vitro* total bone marrow cultures to assess any changes in the IgM concentration in either the femur and sternum bone marrow. In addition, the frequency of plasma cells, in the marrow, was determined using cytodots of bone marrow cells that were stained with antibodies to IgM, IgG, and IgA. Bone marrow-derived stromal cells from sternum were evaluated to determine if, similar to femoral stromal cells, they can maintain plasma cell survival as indicated by

immunoglobulin secretion. This section of results investigates different aged stromal cells to determine if stromal cells from aged mice facilitate more efficient *in vitro* plasma cell niche compared to young stromal cells.

CHARACTERIZATION OF THE PLASMA CELLS FROM FEMORAL AND STERNUM BONE MARROW

To determine the similarities of these two distinct bone marrow sites, I first assessed IgM production in cultures of total bone marrow. Previous observations in the lab found that IgM-secreting plasma cells are present at low frequency in young mice, and the frequency increases with age. This phenomenon is easily measured by ELISA for IgM. The concentration of secreted IgM is a reasonable reflection of the abundance of plasma cells in a bone marrow sample. Bone marrow cells were cultured at two different cell densities (100,000 and 250,000), and supernatants were collected every twenty-four hours for three days. Two concentrations of bone marrow cells were chosen for a specific reason. Obviously, a single concentration would determine if the two sites were equivalent in IgM production. However, it would be possible that a lower bone marrow cell concentration would have a detectable difference in antibody production, via intrinsic immunoglobulin regulation, without an actual decline in plasma cells. Instead, this antibody difference may reflect a potential rate limiting factor that would be

compensated for when total bone marrow cells are cultured at a higher concentration. Thus, the two cell concentrations were used to elucidate between these possibilities.

As I initially predicted, at the lower cell concentration (100,000 cells per well), there was a difference in detectable IgM secretion between aged femur/tibia and sternum bone marrow (Figure 6A). At day one, femoral bone marrow had an elevated concentration of antibodies compared to sternum that quickly declined by forty-eight hours. In contrast, sternum bone marrow had a more consistent antibody production. This pattern of antibody production could represent an increase in antibody producing cells in the sternum, or it could represent a similar frequency of plasma cells but with a difference in the microenvironments. To address this question, bone marrow cultures were established at a higher cell concentration (250,000 cells per well) and IgM concentrations were assessed by ELISA. If the small increase in IgM concentration over time was due to an increase in IgM producing plasma cells in the sternum, then a proportional increase in IgM in the sternum compared to the femur would be expected (Figure 6B). The results demonstrate that when bone marrow cells are cultured at the greater concentration, an equivalent amount of IgM was detected in the culture supernatants from both marrow sources at twenty-four hours and throughout the three day period (Figure 6). These results suggest there may be a similar frequency of plasma cells in both marrow sites.

Plasma cell frequency was then directly assessed by staining for intracellular immunoglobulin. This approach evaluates a representative bone marrow population and identifies the desired cells with an immunoglobulin-specific antibody (anti-IgM/A/G) with a fluorochrome. Cytodots were generated by centrifuging 250,000 total bone marrow cells to slides. Cells were obtained from young (2-3.5 months) and old (18-24 month) wild-type BALB/c femur and sternum bone marrow. Cells were then fixed with 95% ethanol/ 5% acetic acid and stained with a goat anti-mouse IgM/A/G antibody, conjugated to FITC (Figure 7A). Total cells were viewed by phase contrast and the numbers of plasma cells (which are FITC+) were scored using the fluorescent microscope. The frequency of plasma cells was calculated by dividing the number of plasma cells by the number of overall cells counted (which were a minimum of 1,000) and multiplied by one hundred. If the bone marrow sites regulate the specific antibody production capabilities of plasma cells, then I expect to see a distinct difference in the frequency of IgM/A/G positive plasma cells (Figure 7A). However, when cytodots from both bone marrow locations were assessed for plasma cells, there was only a slight elevation in the sternum (Figure 7B). Additionally, this elevation was not significant ($p=0.081$ (young) and 0.133 (old)). This approach did allow us to determine that regardless of the location, there was a significant increase of IgM/A/G positive plasma cells in the aged marrow ($p=0.002$). This strengthens our previous unpublished data that

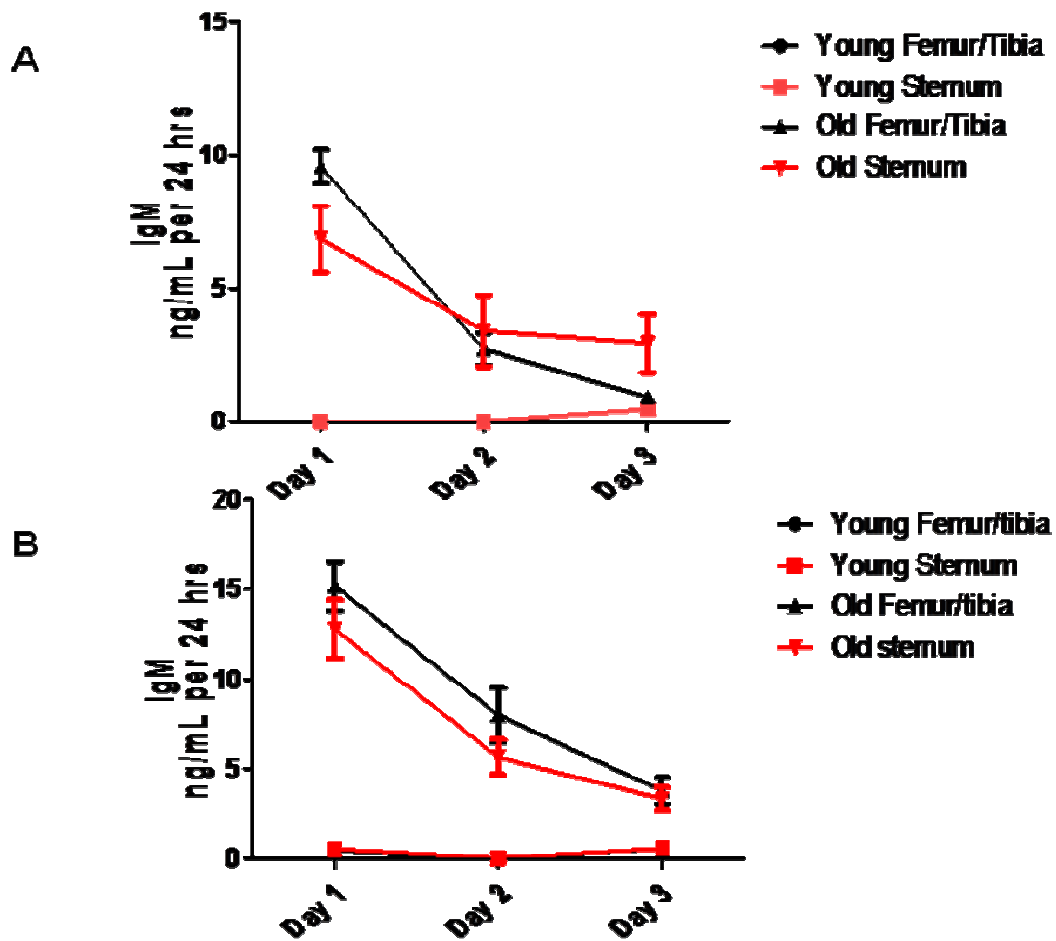
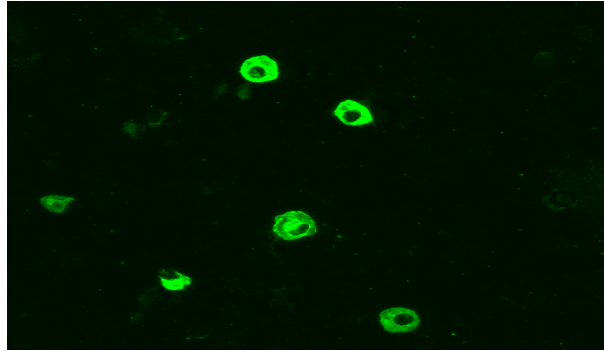


Figure 6: IgM production in total bone marrow cultures. Total bone marrow cells were isolated from young (6 weeks) and old (18 months) wild type femur/tibia or sternum bone marrow. Cells were isolated and counted by trypan blue exclusion and were incubated at either 100,000 (A) or 250,000 (B) cells/well, in triplicate wells. Supernatants were harvested and replaced with fresh media daily. Supernatants were assessed for total IgM production by ELISA. N= 3 for old mice (points are averaged and assessed by SEM) and N=1 for young mice. Unpaired t-test, $p=0.9973$ (100,000 cells) and $p=0.7128$ (250,000 cells). These statistics were done on the overall line per each sample group. Statistics for (A) day one, by unpaired t-test revealed no significance ($P=0.0673$)

A



B

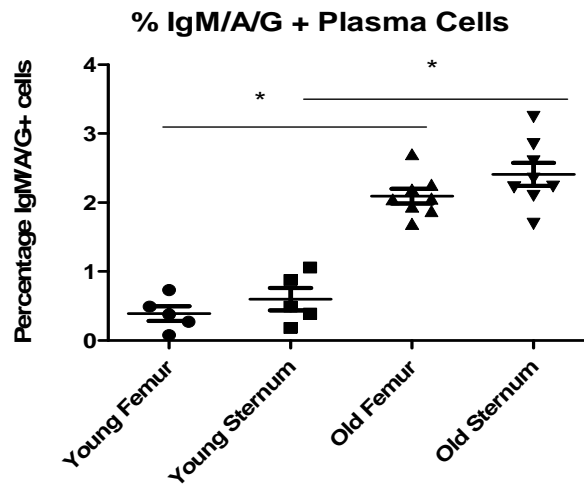


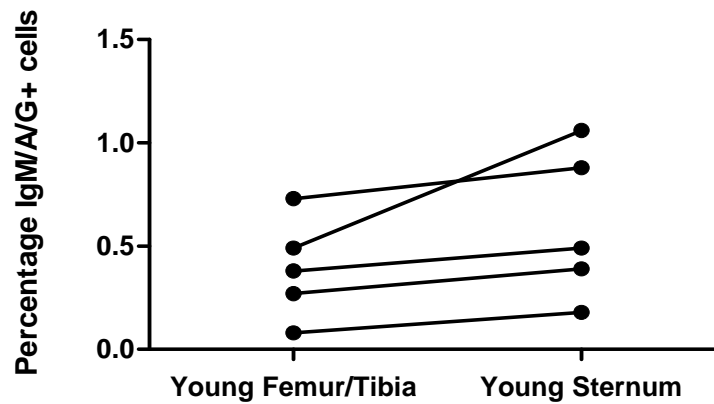
Figure 7: Frequencies of plasma cells in femur and sternum bone marrow from young and old mice. Plasma cells were detected by fluorescence and confirmed by morphology (A). 250,000 bone marrow cells were spun onto a slide and were stained with IgM/A/G-FITC antibodies. Paired t-test statistically assessed the differences in age-matched mice ($p = 0.081$, young; $N=5$) and ($p=0.133$, old; $N=8$) and unpaired t-test were used to assess differences between young and old for each bone marrow type ($*p=0.002$) (B).

suggests there is an accumulation of plasma cells in the marrow of aged mice, compared to the frequency found in young bone marrow. This finding also demonstrates that this accumulation is a universal phenomenon for the bone marrow and suggests that the signals and cellular composition that maintain the plasma niches may indeed be similar in both the femur and sternum.

In order to determine if the increase in sternum plasma cells (although not significant) represented a consistent trend, plasma cell frequencies were compared in individual mice using immunofluorescence staining for IgM/G/A. If the increase in sternum plasma cells represented a common trend, then I expected to see a consistent increase in plasma cell frequency in the sternum compared to the frequency in the femur of each individual mouse. As expected, sternum plasma cell frequencies regularly [92% of the time (11/12 events)] were approximately 0.1-0.2 % higher than those in femur, regardless of the host's age (Figure 8). These findings indicate that although the antibody concentrations became equivalent at the higher cellular concentration (250,000 cells per well), there appears to be an increased frequency of plasma cells in the sternum on a mouse to mouse basis.

In summary, the plasma cell compartments appear to be equivalent in regard to IgM production; however, the sternum houses a higher frequency of total plasma cells secreting IgM, IgG, and IgA. This suggests that these two bone marrow sites function

A.



B.

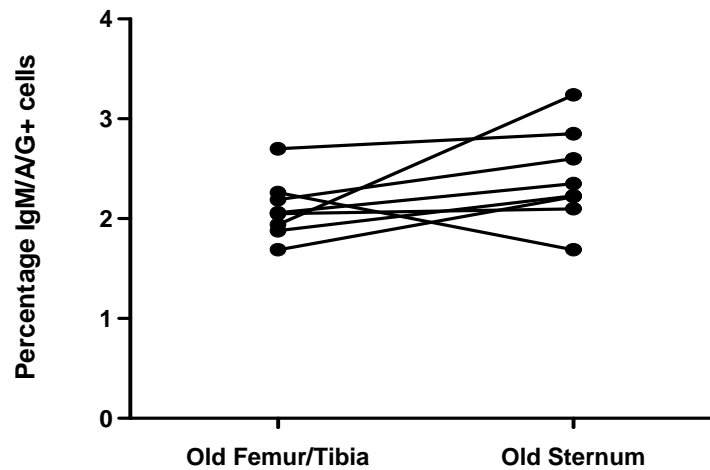


Figure 8: Frequencies of plasma cell and correlation within a single mouse. Total bone marrow cells were isolated from (A) young (6-8 weeks; N=5) and (B) old (18-24 month; N=7) femur and sternum wild type BALB/c mice. 250,000 cells were centrifuged to each slide and were stained with goat-anti-mouse IgM/A/G antibody. The investigator was blinded to the age and organ to not bias the results. Frequency was assessed by taking the number of plasma cells and dividing it by the total number of cells counted times one hundred. Each line represents the frequencies of plasma cells in an individual mouse.

similarly in the mouse, but may have individual plasma cells with different functional capabilities. The results may also illustrate a preference for the sternum to harbor more IgA and IgG producing plasma cells than the femur. Without assessing IgA and/or IgG production separately, it remains to be determined if the overall production of immunoglobulin is equivalent or different between the two bone marrow derived plasma cell subsets.

ARE FEMUR AND STERNUM PLASMA CELLS INTRINSICALLY SIMILAR IN REGARD TO *IN VITRO* SURVIVAL?

Now that I have characterized the relative abundance of plasma cells that reside in the femoral and sternum bone marrow, based on the type of immunoglobulin they produce, I next sought to determine if these two populations of plasma cells are intrinsically similar in regard to *in vitro* survival. In our system, plasma cell survival on stromal cells was assessed by total IgM production. Stromal cells from long-term bone marrow cultures were chosen as the feeder cells because prior experiments demonstrated that stromal cells are capable of supporting plasma cells survival *in vitro* (62). Aged femur stromal cells were isolated by sorting via FACS (see materials and methods section for additional details). One thousand plasma cells, isolated from either aged femoral or sternum bone marrow, were cultured on duplicate feeder cell layers. Previous data (Figures 6-8) illustrated that it is possible that sternum plasma cells, although at a higher

frequency, produced equal amounts of IgM than femur plasma cells. If an intrinsic difference exists between femur and sternum plasma cells, then I expected to see a decline in antibody production by sternum plasma cells, compared to femur cells. When one thousand plasma cells were cultured from either site, there was an initial and consistent decline in sternum produced IgM in the culture supernatant compared to that from femur plasma cell cultures (Figure 9). However, both populations survived well until day twenty one and then diminished rapidly, at the same rate, between days twenty one and twenty four. This finding suggests that plasma cells, regardless of bone marrow site, survive similarly *in vitro*. Although both populations of plasma cells had similar survival profiles, the femoral plasma cells had an increased secretion of IgM compared to sternum. The difference in overall immunoglobulin secretion suggested that either femur-derived stromal cells do not support sternum plasma cells, or that this population of sternum plasma cells contains fewer IgM producers. To confirm that both plasma cell subsets were surviving at equivalent rates, microscopy was used two times a week to monitor cell death by morphology and overall debris. By microscopy, both cell types appeared to have had similar survival (personal observation).

Overall, these data suggest that the increased frequency of IgM/A/G positive plasma cells in the sternum (figure 7 and 8) has a slightly decreased amount of IgM

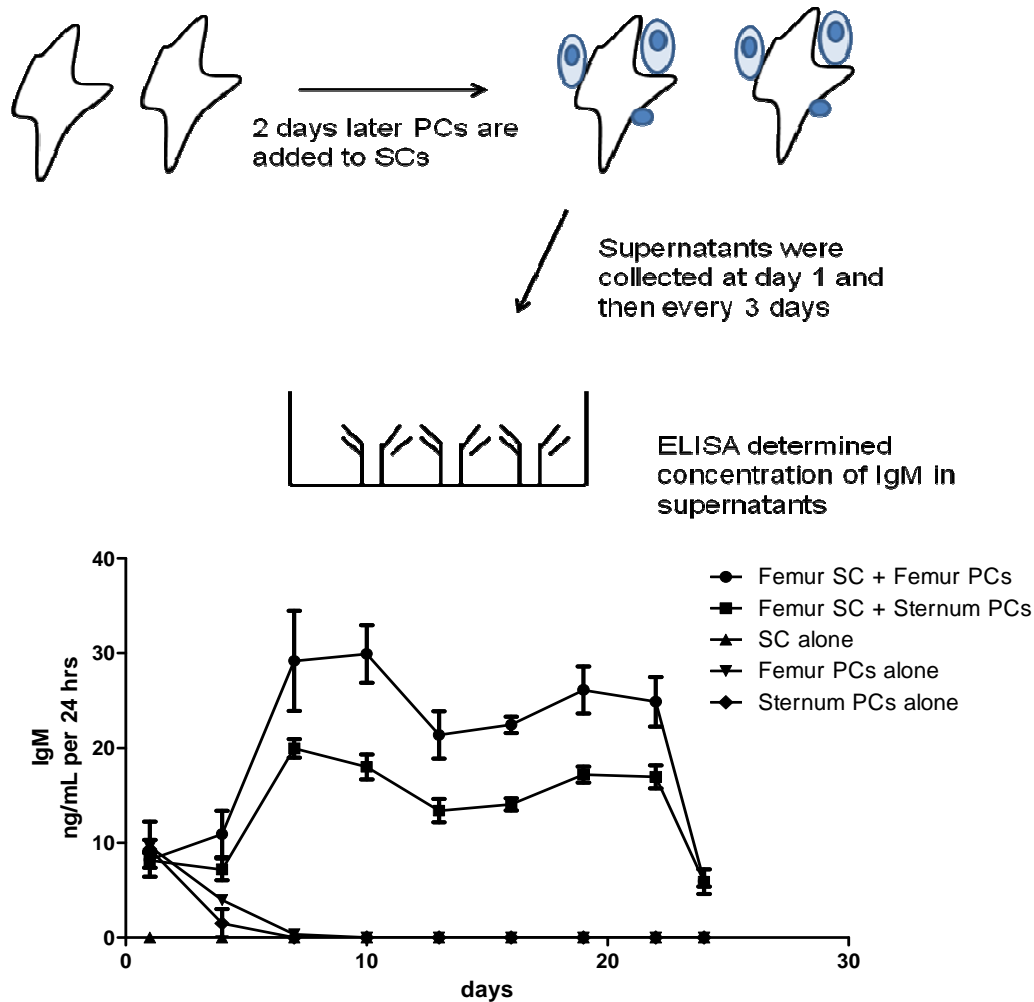


Figure 9: Comparison of *in vitro* survival of femur and sternum plasma cells on aged femoral stromal cells. Stromal cells were isolated from long-term bone marrow cultures that were generated from aged femur cells. Stromal cells were sorted directly into single 96-wells at a density of 10^4 cells/well. Two days later, three aged mice were sacrificed and bone marrow was flushed from the femur and sternum to isolate plasma cells. Plasma cells were first enriched by magnetic bead isolation using rat anti-mouse CD138-PE and anti-PE beads. Next, plasma cells were sorted by FACS, based on size and fluorescence, and 10^3 cells were added to each well occupied with stromal cells. Supernatants were collected at day one and then every three days for twenty-four days and immunoglobulin concentrations were determined by ELISA. (N=1 experiment)

production when compared to femoral plasma cells. In addition, *in vitro* survival of plasma cells appears to be stable when co-cultured on aged femoral stromal cells. With similar survival patterns and IgM production, the differences in these two populations may depict skewing towards IgA and IgG in the sternum. Overall, the results from the plasma cell frequency and survival experiments may represent intrinsic differences between plasma cell subsets that correlate with an *in vivo* difference in the overall function of the host's multiple populations of plasma cells.

ARE FEMUR- AND STERNUM- DERIVED STROMAL CELLS EQUIVALENT IN MAINTAINING PLASMA CELL SURVIVAL *IN VITRO*?

The previous experiments suggested that despite the increased frequency of IgM/A/G plasma cells in the sternum, the overall production of IgM from these plasma cells, isolated from unimmunized mice, is similar. Thus far, all the *in vitro* experiments were done using femoral microenvironmental cells (stromal cells). The question remains if various stromal cells are similar or different in regard to *in vitro* plasma cell survival. I attempted to address this question by comparing the response of femoral plasma cells on either femur- or sternum-derived stromal cells. I chose femoral plasma cells, instead of plasma cells from sternum for two reasons. Not only has our lab used femoral plasma cells in numerous studies, which allows us to take advantage of comparisons with

previous data, but also, the isolation of femoral plasma cells is more routine compared to sternum.

As shown in Figure 9, stromal cells from aged femur are capable of maintaining plasma cell survival. I assessed plasma cell survival on either young (6 week) or aged (15 month) femur- or sternum-derived stromal cells. Stromal cells were isolated from long-term bone marrow cultures maintained in our lab. After they were sorted and aliquoted into 96-well plates seventy-two hours earlier, sorted femoral plasma cells were added to each well and supernatants were collected to assess plasma cell IgM production. The first question addressed was, “are aged femoral stromal cells comparable to young stromal cells in ability to maintain plasma cell survival *in vitro*?” Using plasma cells from a single isolation, I could assess if the various aged stromal cells are capable of maintaining their survival. If the age of femoral stromal cells positively affects plasma cell survival, then I expected to observe increased immunoglobulin in supernatants from plasma cell cultures with aged stromal cells compared to ones cultured with young stromal cells. As predicted, the concentration of antibody was increased and an elevated level of IgM was maintained throughout the experiment when plasma cells were co-cultured with stromal cells from aged mice (Figure 10). Considering that prior experiments utilized young stromal cells, we were surprised that cultures containing young stromal cells had a rapid decline in IgM production after day one (62). The

decline in IgM continued for the duration of the experiment, and IgM concentrations were only slightly elevated when compared to supernatants from plasma cells alone. This suggests that young stromal cells provide less support to plasma cells than aged stromal cells. My data support prior findings that there is an accumulation of plasma cells in aged femur compared to young and suggest that this accumulation may be due to the maturation of stromal cells. The enhanced support of plasma cells by aged femoral stromal cells led me to question if this was a common trait of all bone marrow stromal cells. Since there was an accumulation of plasma cells in both femoral and sternum bone marrow sites, I predicted there would also be a similar age-related difference in plasma cell maintenance using stromal cells isolated from sternum. If this trait of providing adequate *in vitro* niches is common to all bone marrow stromal cells, then I expect to see an age-related increase in plasma cell survival, as assessed by IgM production. These sternum-derived stromal cells were generated from the same mice as the femur experiment and co-cultured with isolated plasma cells from the same sort (Figure 10). Similar to previous observations, there was a sharp decline in daily IgM production in all experimental groups between twenty four and ninety six hours (Figure 11). However, unlike stromal cells derived from femur, young and aged sternum stromal cells both maintained IgM production, to similar degrees, for the duration of the experiment. It is of importance to note that when these stromal cells were cultured alone, there was no IgM

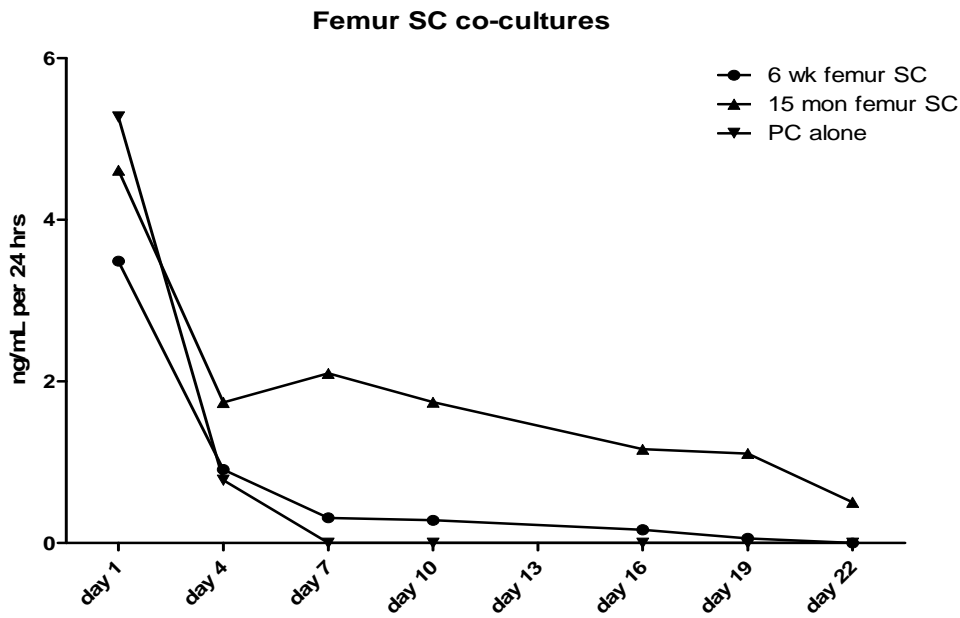


Figure 10: Femur stromal cell age and its role in maintaining plasma cell survival *in vitro*. Young (6 week) and old (15 months) stromal cells were cultured with aged (18-24 months) femoral plasma cells for twenty-two days. Supernatants were collected every three days after the initial set-up (day one). These supernatants were used to assess IgM production by ELISA. Unpaired t-test (of overall age grouped lines), $p=0.113$. $N=1$ experiment.

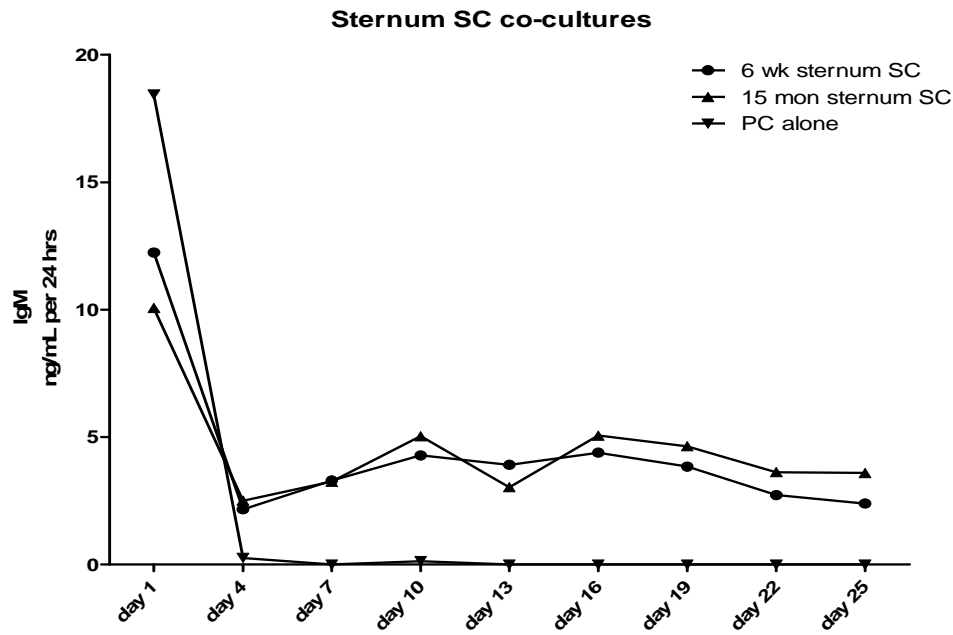


Figure 11: Sternum stromal cell age and their role in maintaining plasma cell survival *in vitro*. Young (6 week) and old (15 months) sternum stromal cells were cultured with aged (18-24 months) femoral plasma cells for twenty-five days. Both the stromal cell and plasma cell sorts were the same as used in Figure 9. Supernatants were collected every three days after day one. Supernatants were used to assess IgM production by ELISA. Unpaired t-test $p=0.8989$ (of overall age grouped lines). $N=1$ experiment.

production detected, demonstrating that there is no plasma cell contamination during stromal cell sorts (data not shown). Although this experiment was only done once, this preliminary result suggests that cultured stromal cells derived from different bone marrow sites may act uniquely. This finding, if reproducible, would help researchers focus on the mechanisms that allow these sites to be different.

The stromal cell data illustrate that (1) aged stromal cells from either marrow location can maintain IgM production for over twenty days, in this experiment, and (2) stromal cells from young sternum support plasma cell survival better than those from young femur (Figures 10 and 11). This led to the last question in regard to the difference in the microenvironments of both sites. Since aged stromal cells (regardless of location) are capable of maintaining plasma cells, is there any difference in this process? The expectation would be that if aged stromal cells had a universal trait in maintaining plasma cell longevity, then I expect to see similar production of IgM from plasma cells cultured on stromal cells regardless of site of origin. To test this idea, stromal cells were prepared from long-term bone marrow cultures generated from the femur or sternum of an individual mouse. These stromal cells were cultured for three days before plasma cells, pooled from three mice, were co-cultured with these stromal cells. Regardless of the origin of the stromal cells in the culture, plasma cells produced roughly the same concentration of IgM over a twenty four hour period (Figure 12). This suggests that

while host's age affects the ability of stromal cells to support plasma cell survival in vitro, the stromal cells origin does not play a role in this process. Although these co-culture experiments hint at mechanisms that may regulate plasma cell survival and immunoglobulin secretion, it is important to note that the detected amount of IgM in the supernatants varied greatly (IgM concentrations were 10 times greater in Figure 12 compared to Figure 10). Without repeating these experiments, we are unable to conclude if this is due to an experimental error, or support the idea that plasma cells are not uniform in their immunoglobulin production, on a cell to cell basis.

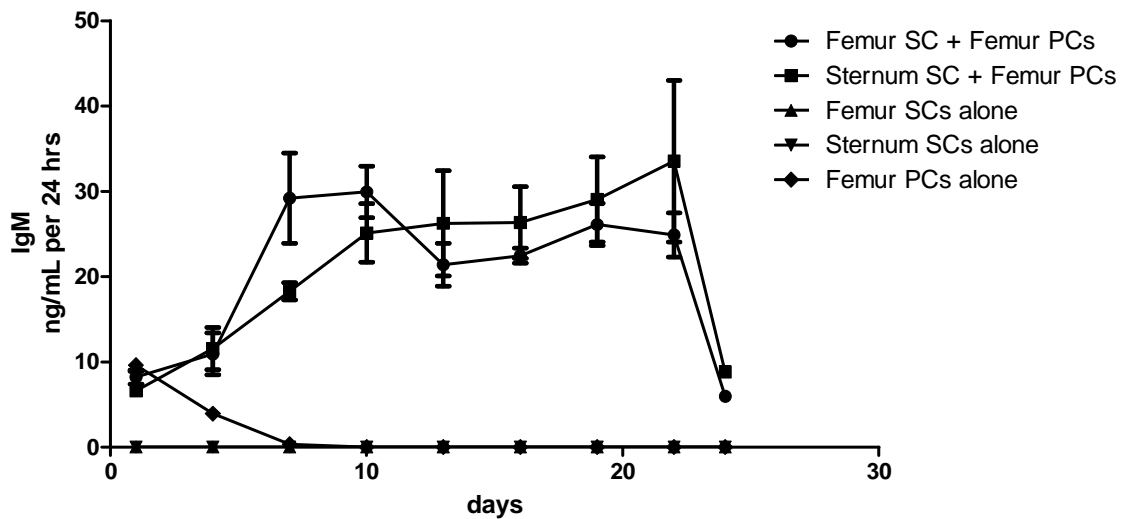


Figure 12: Aged stromal cells, from femur or sternum, and their capability in maintaining plasma cell survival *in vitro*. Stromal cells were isolated from both the femur and the sternum of an aged (18 months) mouse. 10^4 stromal cells were cultured with 5×10^3 femoral plasma cells from aged mice. Supernatants were collected every three days and IgM concentrations were determined by ELISA. Unpaired t-test $p=0.8733$. Each time point is an average of 4 readings and error bars depict SD. N=1 experiment.

SUMMARY

Research involving plasma cells and their appropriate niches gained interest in the last decade. Much work has been done to investigate how plasma cells and their niches work together to elicit protective immunity. However, this research has most often utilized femoral bone marrow and has not addressed whether differences exist in marrow sites in regard to either the frequency or maintenance of plasma cells.

My work, however, highlights the importance of observing multiple sites. Since cortical and membranous bones develop differently, the bone marrow of these two sites may be influenced to develop differently as well. My work has shown that there is a trend toward elevated frequencies of plasma cells in the sternum compared to the femur. My preliminary data demonstrated that sternum plasma cells, as a population, may produce less IgM. To determine if the plasma cell niches were different in these two bone marrow sites, I next assessed the ability of the stromal cells to support plasma cells *in vitro*. My preliminary work illustrates that unlike femur, sternum does not appear to have an age-related increase in the ability to support plasma cell survival *in vitro*. However, aged stromal cells, regardless of their origin, are equally capable of maintaining plasma cell survival in culture. These data support the notion that despite the generally universal mechanisms supporting humoral immunity, there are some characteristics that are unique to particular host locations (Figure 13).

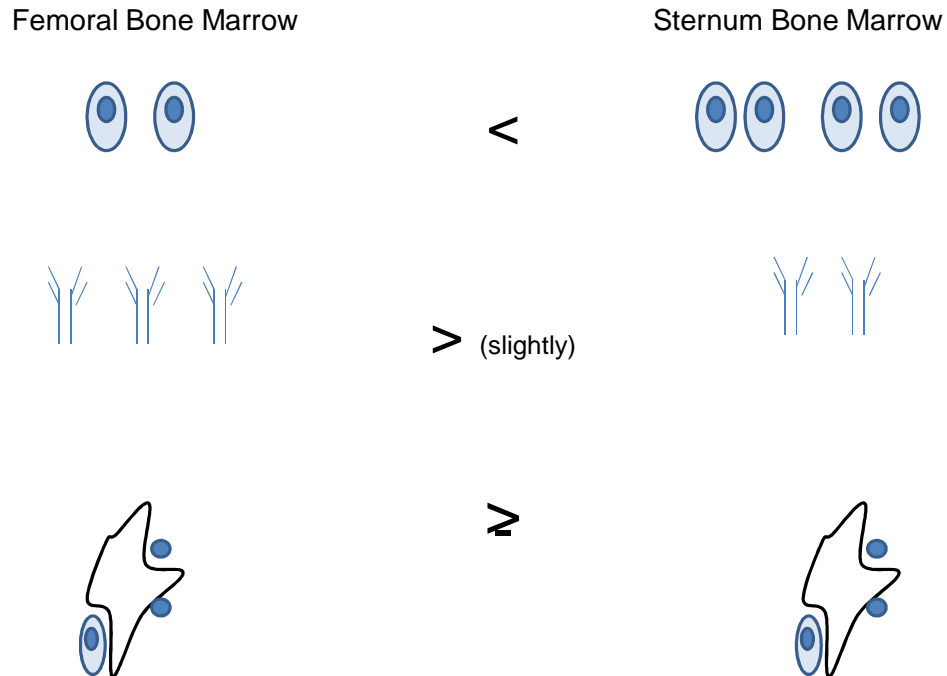


Figure 13: Influence of bone marrow origin on plasma cells and their function.

- The percentage of IgM/A/G positive plasma cells were elevated in the sternum compared to the femur of the same mouse.
- In vitro IgM production was only slightly increased from isolated femoral plasma cells co-cultured on femoral stromal cells, compared to sternum plasma cells.
- These two findings suggest that either of these two locations have similar IgM positive cells, and differences reside in IgA and IgG producing cells, or femoral plasma cells are capable of secreting more IgM than sternum plasma cells.
- Stromal cells from the murine femur have a higher capability of supporting plasma cell survival in vitro, suggesting that stromal cells may not be uniform in their production and expression of plasma cell growth factors and surface molecules.

CHAPTER IV

RESULTS- EXAMINATION OF APRIL AND ITS RECEPTOR TACI IN YOUNG AND OLD BONE MARROW

Chapter three focused broadly on the bone marrow microenvironment and sought to determine if there is one common microenvironment, or whether microenvironments differ depending on their origin. The data suggested that the microenvironments were similar in maintaining plasma cell survival *in vitro*, while sternum had an increased frequency of plasma cells. However, both microenvironments had an overall accumulation of plasma cells in the aged marrow, demonstrating a shift to mature B cell phenotypes as the host ages. This chapter will focus on signals provided by the microenvironment that affect the B cell compartment. In particular, Chapter Four will assess expression of a signaling molecule, APRIL and one of its receptors, TACI, for changes with age.

The bone marrow not only provides cellular contact, which is essential for the survival of many cell types, but also produces various growth factors for the resident cells (5, 6, 9, 11, 24, 33, 35, 60, 62, 65, 66, 72, 84). These factors provide signals to cells, driving their maintenance, functionality, and survival in the bone marrow. In particular, B cells require these signals for their development and production of antibodies. One

specific signal is provided by the interaction between soluble APRIL and its cognate receptor, TACI, which is found on all peripheral B cells, including plasma cells (29, 54, 55, 74). APRIL is produced by multiple bone marrow residing cells, including dendritic cells and activated B cells (54). Once APRIL binds to TACI, this signal activates various signaling pathways in B cells, by activating signaling molecules and transcription factors such as NF κ B and JNK (54). Activation leads to the expression of genes involved in B cell development and function.

EXAMINATION OF APRIL TRANSCRIPTS IN THE MURINE BONE MARROW AND SPLEEN

To determine if a change in the expression of APRIL contributes to the shift to mature B cells in the aged bone marrow, I assessed APRIL transcripts in various aged murine femoral and sternum bone marrow samples. By first comparing the APRIL expression in young and old bone marrow, the question can be asked as to whether APRIL expression changes with age in these microenvironments. Freshly isolated femoral or sternum bone marrow was pelleted, at a density of 1×10^6 - 10×10^6 cells/pellet. I isolated RNA from the pellets and cDNA was synthesized. Real-Time PCR analysis compared the expression of APRIL to the constitutively expressed housekeeping gene HPRT. Since APRIL transcripts have been previously detected in the spleen, spleen was used as 1) a positive control for our APRIL primers, and 2) a variable,

to determine if APRIL expression changes in the secondary lymphoid tissue with age (14). If the shift to mature B cells in the aged bone marrow is due to APRIL expression, then I expect to detect an elevated expression of APRIL in old bone marrow samples, when compared to young samples.

As predicted, when femoral bone marrow was assessed for APRIL transcripts, there was a significant increase in APRIL in the aged femoral bone marrow, compared to young (Figure 14). This finding suggests that the aged femoral bone marrow is primed to contribute more APRIL signals than young femoral bone marrow. However, without assessing APRIL at the protein level, I cannot conclude if aged femoral bone marrow produces more signaling protein than young.

To determine if sternum also demonstrates this trend in producing more APRIL transcripts in aged bone marrow, I isolated sternum bone marrow from the same mice as in the previous experiment (Figure 14). Since sternum also exhibited an increased frequency of plasma cells in its aged bone marrow microenvironment (Figure 6), I predicted that the aged sternum bone marrow would contain an increase in APRIL transcripts compared to young. Similar to what was observed in the femur, there was a significant increase in APRIL transcript expression in the bone marrow from sternum of old mice when compared to young (Figure 15). The data suggest that the aged primary

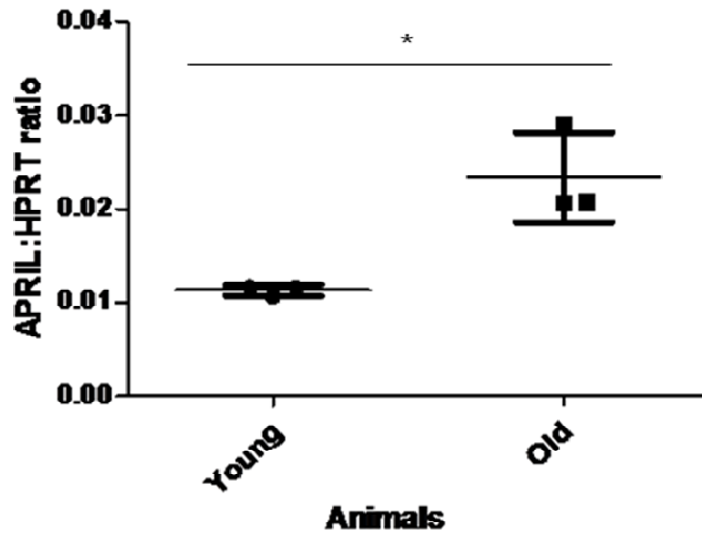
APRIL:HPRT ratio in young and old Femur BM

Figure 14: Expression of APRIL to HPRT in femoral bone marrow. Young (8 weeks) and old (20 months) femoral bone marrow was assessed for APRIL expression by Real-Time PCR. Results are given as a ratio between APRIL and the housekeeping gene HPRT. Unpaired t-test, $P = 0.0122$. $N = 3$ mice per age group.

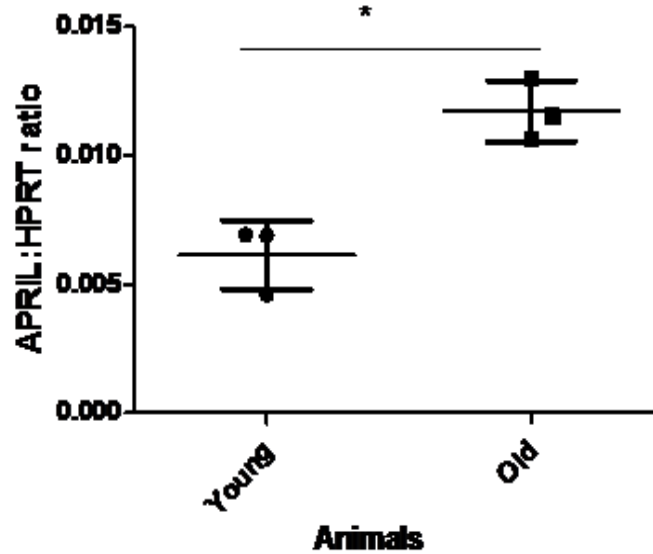
APRIL:HPRT ratio in young and old Sternum BM

Figure 15: Expression of APRIL to HPRT in sternum bone marrow. Young (8 weeks) and old (20 months) femoral bone marrow was assessed for APRIL expression by Real-Time PCR. Results are given as a ratio between APRIL and the housekeeping gene HPRT. Unpaired t-test, $P = 0.0056$. $N = 3$ mice per age group.

lymphoid tissue is more equipped to provide the growth/maintenance APRIL signal than the primary lymphoid tissue found in young mice. This finding could represent a mechanism by which the bone marrow facilitates the conversion of the B cell repertoire from an immature to a mature phenotype.

Thus far, the data suggest that the expression of APRIL transcripts increases in the bone marrow as the mouse ages. However, a major site of mature B cells and the developing plasma cells (plasmablasts) is the secondary tissue, in particular the spleen. Previous research has demonstrated the importance of APRIL in the spleen and how APRIL's absence causes the decline of both mature B cells and antibody production (29, 68). Since spleen has been used as a positive control for APRIL expression previously, we already know that this organ has detectable transcripts in young mouse samples. However, research has not assessed the basal levels of APRIL transcripts in both young and old spleens, and whether they remain constant or vary with age. My assumption was that since there is an increase in plasma cells in the aged host, there would be a consistent or elevated amount of APRIL transcripts in the old spleen when compared to young spleen. When assessing APRIL expression by Real-Time PCR, a consistent, significant elevation in the ratio of APRIL to HPRT in the old (20 months) spleen samples was observed (Figure 16). This increase supports previous findings implementing the importance of the secondary lymphoid tissue for the development, survival, and

maintenance of the mature B cells. It's possible that this increase in available transcripts correlates with an increase in APRIL protein, which can facilitate these functions in the spleen.

The data in Figures 14 through 16 assessed the expression of APRIL in unimmunized young and old mice. Our earlier findings (see previous chapter) suggest that, at least in regard to plasma cells, there tended to be a difference in cellular frequency between femoral and sternum bone marrow. This led me to question whether the expression of APRIL transcripts also varies between these two bone marrow sites. To determine if the two bone marrow sites harbor equivalent concentrations of APRIL transcripts, total bone marrow cells were isolated from the femur and sternum of the same mouse. These cells were then lysed to obtain cellular RNA and Real-Time PCR was utilized similar to prior experiments. The prediction was that if the expression of APRIL affects the overall maintenance of plasma cells in the bone marrow, then an elevated level of APRIL was expected in the sternum compared to the femur of the same mouse. Additionally, if the previous propositions were correct, then this prediction would be evident in both the young and old sternum compared to age-matched femur, due to the increase in plasma cell frequencies in the sternum, regardless of age (Figure 7). However, when observing young femoral and sternum bone marrow, the APRIL/HPRT

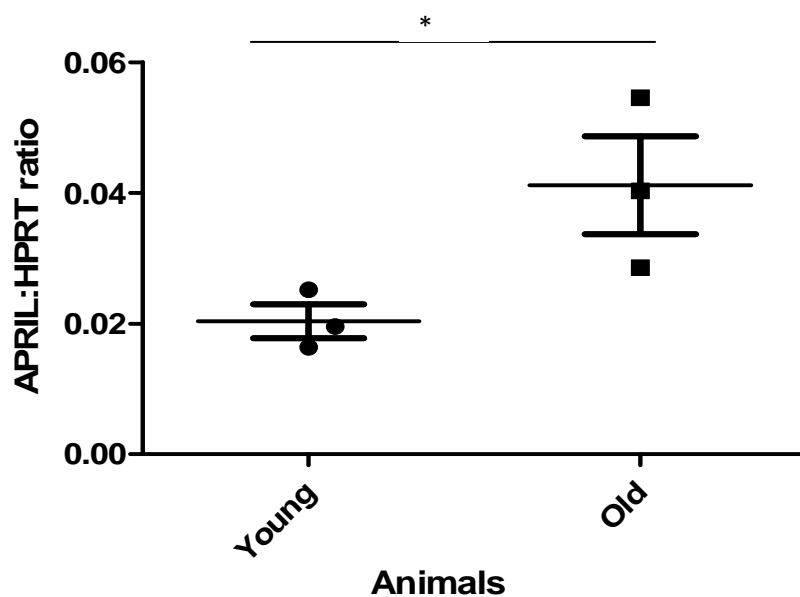


Figure 16: Expression of APRIL to HPRT in spleen. Young (8 weeks) and old (20 months) spleen was assessed for APRIL expression by Real-Time PCR. Results are given as a ratio between APRIL and the housekeeping gene HPRT. Unpaired t-test, $P = 0.0356$. $N = 3$ mice per age group.

ratio was only slightly elevated in the sternum compared to femur and was not significant (Figure 17 A). This trend was also apparent in the old femoral and sternum samples; however, there was more variability in APRIL transcript expression between samples (Figure 17 B). It remains to be determined if this slight elevation in APRIL transcripts generates a difference in APRIL protein.

In summary, APRIL is produced by numerous cells in the host, which allows for APRIL's function to be displayed in both the primary (bone marrow) and secondary (spleen) lymphoid tissues. Examination of APRIL transcripts revealed an elevation in both the primary and secondary lymphoid tissue in aged mice, which were typically around twenty months of age. To determine if the production of APRIL is similar in both femoral and sternum bone marrow, I performed Real-Time PCR on bone marrow isolated from the same mice. These experiments demonstrated that there is similar production of APRIL in both marrow sites. This section provided additional insight into the expression of APRIL, as well as illustrating a potential mechanism that contributes to the accumulation of mature B cells in the older hosts.

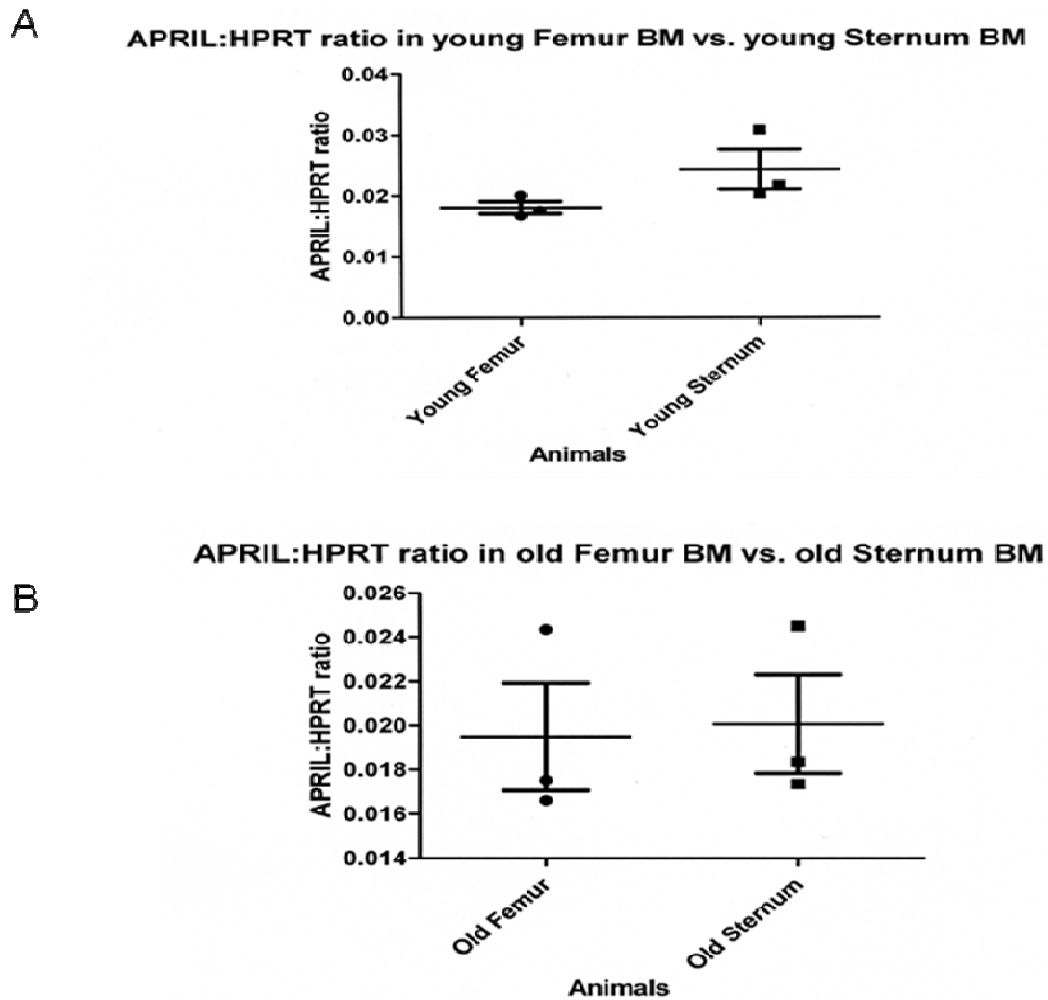


Figure 17: Comparison of APRIL:HPRT ratios in the femoral and sternum bone marrow. Young (8 weeks) and old (20 months) femoral and sternum bone marrow was isolated from the same mouse and assessed for APRIL expression by Real-Time PCR. Results are given as a ratio between APRIL and the housekeeping gene HPRT. Unpaired t-test $P=0.2419$ (young) and 0.1109 (old). $N=3$ experiments per age group. **Important to note: these experiments were done separately than experiments for figures 14 and 15. *** See appendix for raw data chart on these results

EXAMINATION OF TACI EXPRESSION ON VARIOUS B CELL SUBSETS

Cells respond to stimuli based on the engagement of a signaling molecule (ligand) to its cognate receptor. As previously determined, APRIL is a signaling molecule produced by the microenvironment. APRIL can bind to TACI, which is located on all peripheral B cells and plasma cells (56, 68). Upon engagement, a signaling cascade elicit gene expression important in B cell development and function (7, 13, 54, 56, 68).

Research has determined the cell subsets that express TACI (28, 55, 56, 74, 89). However, current research has not examined the expression of TACI on B cells from various aged naïve mice. It is important to determine if elevated expression of APRIL in aged mice (Figures 14-17) correlated with up-regulation of one of its receptors, TACI. This helps clarify whether TACI is the receptor preferred in the aged host. To determine if TACI expression changed on any of the marrow B cell subsets with increased age, bone marrow cells were flushed from the femoral bones of both young (5-8 weeks) and old (18-24 months) naïve mice. To discriminate B cell subsets, these cells were stained with antibodies to B220 (a surface marker to identify the various subsets of B cells), CD138 (Syndecan-1, a proteoglycan found on the surface of plasma cells) and TACI. FACS was used to analyze the cells in the bone marrow, based on the expression of these three markers. Specifically, cell samples were first gated for viable cells, based on their forward and side scatter (FSC and SSC). Events that fell to the far left corner with

extremely low FSC and SSC were considered dead cells or cellular debris. In the viable cell gate, lymphocytes were gated based on low to moderate FSC and low SSC, since a lymphocyte is typically between seven and ten micrometers in size (Figure 18).

Lymphocytes were selected and segregated based on B220 expression (Figure 19). B220 expression increases as a B lymphocyte matures. B cells that express low levels of B220 are considered a early Pro-B cell, while moderate expression of B220 identifies either a late Pro-B, Pre-B or immature B cells. Lastly, high expression of surface B220 is used in concert with additional B cell markers (like surface IgM (BCR) and IgD) to identify more mature B cell subsets. A B220 negative cell, however, could represent either a non B-cell or a differentiated plasma cell. To separate these two distinct populations, the glycoprotein, CD138, was utilized to identify plasma cells (Figure 20). CD138 (Syndecan-1) is a transmembrane heparin sulfate proteoglycan important in cellular binding and signaling, and has been found to interact with APRIL (38).

Once these subpopulations of B cells were separated in regard to B220 expression, TACI expression was assessed on B cells residing in the murine femoral bone marrow. To assess TACI expression, I calculated the number of each B cell, per population, that expressed TACI on their surface per one hundred thousand total lymphocytes. The prediction is that since APRIL expression increased in the aged bone marrow, the expression of TACI would also increase on B cells from aged mice. When

TACI positive B cells were assessed, there was a uniform increase in the number of cells in all categories found in the aged host (Figure 21). Though a uniform elevation in TACI positive cells, significance was achieved only in the plasma cells (B220 negative and CD138 positive) and mature B cells (B220 high).

These findings demonstrate that the mature B cell populations, in the aged host, are more equipped to respond to the potentially elevated APRIL available in their microenvironment. This could represent a mechanism by which the microenvironment provides signals to facilitate the maintenance of these mature cells *in vivo*. Additionally, these findings could assist future research in determining if an elevation in APRIL is a positive regulator to the increased expression of its receptor, TACI or if these two events are not correlated.

Since the previous results chapter focused on plasma cells and the role the microenvironment played in their survival, I wanted to conclude my thesis by assessing the overall frequency of TACI positive plasma cells. Figure 21 depicts the number of B cells that express TACI on their surface. Though plasma cells accumulate in the aged marrow, their numbers are still relatively small, especially in comparison to other bone marrow residing populations. To assess if the plasma cell population, as a whole, has an up-regulation of TACI on their cell surface, FACS was utilized to gate plasma cells (B220 negative, CD138 positive lymphocytes). These cells were then gated to determine

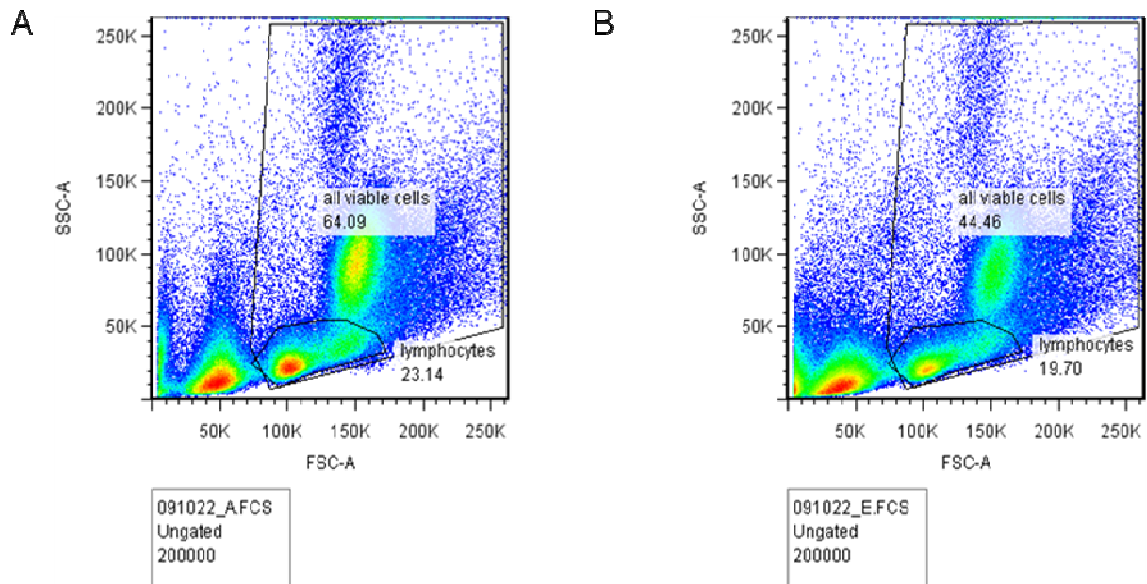


Figure 18: FACS representation of viable cell and lymphocyte gating. These are a representation of FACS gating of a young (A) and an old (B) murine femoral bone marrow. First, all viable cells were gated, based on their forward and side scatter. Then, a lymphocyte gate was drawn, to contain all viable cells that were of small to medium size, to exclude the larger innate immune cells, like dendritic cells and macrophages.

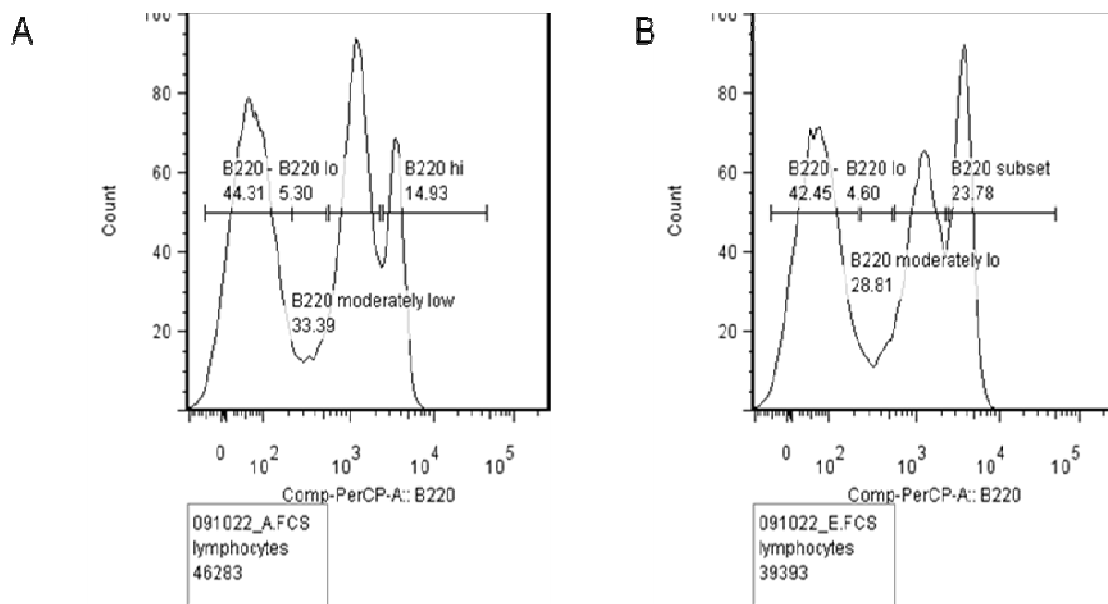


Figure 19: FACS representation of B220 expression on lymphocytes. These are a representation of FACS gating of bone marrow from a young (A) and an old (B) mouse. Lymphocytes were selected (based on gating from Figure 17), and B220 expression was divided into four categories (B220 negative, B220 low, B220 moderate low, and B220 high) based on segregation after fluorochrome compensation performed by FACS.

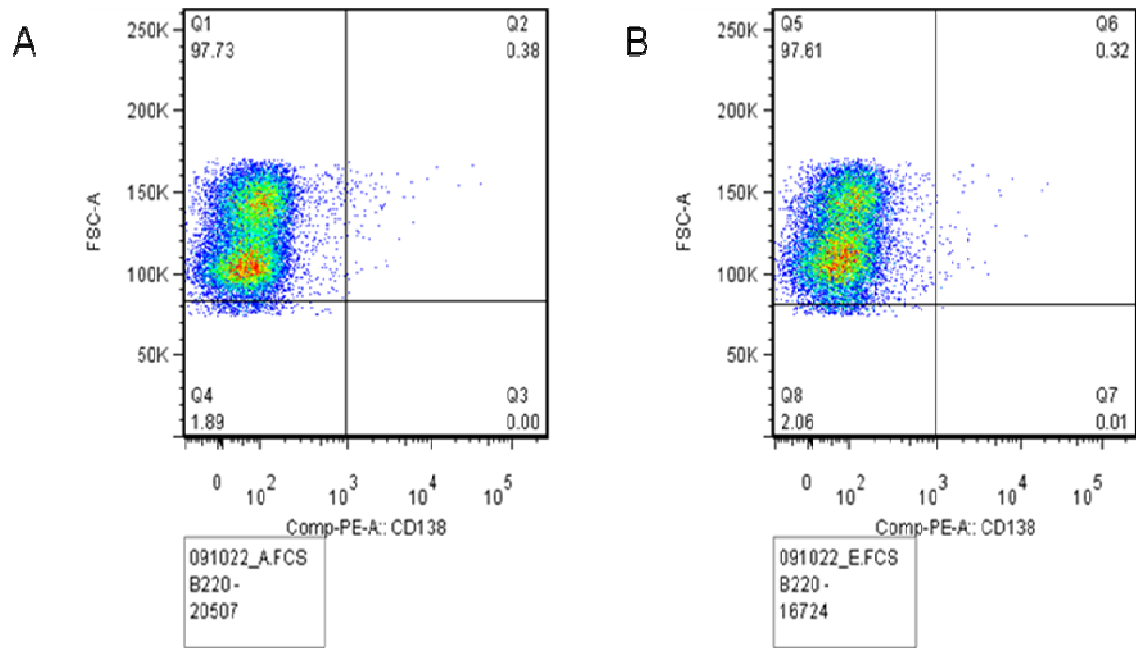


Figure 20: FACS representation of identified plasma cells from young and old bone marrow. These are a representation of FACS gating of a young (A) and an old (B) mouse. B220 negative cells, selected based on expression derived from Figure 19, and were assessed for expression of CD138 on their surface. The expression of CD138 on B220 negative cells with appropriate size and granularity (see figure 18), were considered to be plasma cells. Gating was based on fluorescence minus one compensations and backgating, to ensure this was indeed a plasma cell population.

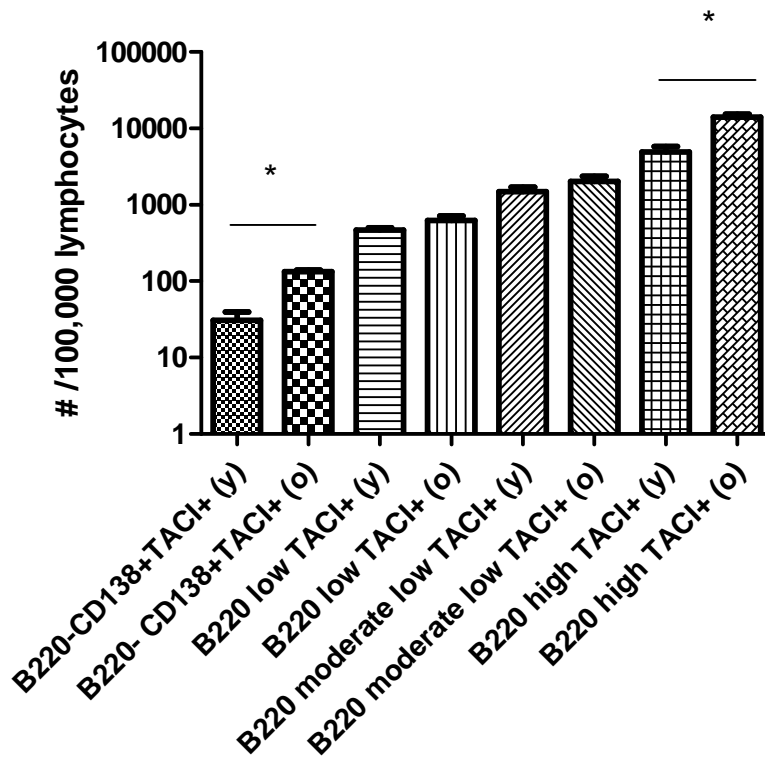


Figure 21: Number of B cells that express TACI on their surface. Based on FACS analysis, the number of TACI positive B cells were calculated and compared between young and old mice. N = 9 mice per young group and 6 per old group. $P = <0.001$ * (B220-CD138+ and B220 high), $P = 0.0948$ for B220 low and $P = 0.1705$ for B220 moderate expression.

the number of TACI expressing cells in this limited sample. TACI positive plasma cell frequencies were calculated by dividing the number of positive cells by the number of B220 negative, CD138 positive cells. The prediction is that if APRIL is important for the maintenance and function of plasma cells in the bone marrow microenvironment, then I expect to see an increase in the frequency of TACI positive plasma cells in the aged mice. As predicted, the frequency of B220 negative, CD138 positive cells that were expressing TACI were significantly elevated in the old mice compared to young (Figure 22).

Similar to the conclusions obtained from figure 21, these results demonstrate that the frequency of plasma cells expressing TACI is increased as the host ages. However, the expression of TACI does not appear to be up-regulated per cell, since both the MFI of TACI is similar on plasma cell from young and old mice (Table 3). Additionally, it appears that there is a population of plasma cells that are negative for TACI, so we can conclude that TACI is not the sole signaling receptor required for plasma cell preservation. Further research is required to identify other signaling pathways critical for these processes to occur.

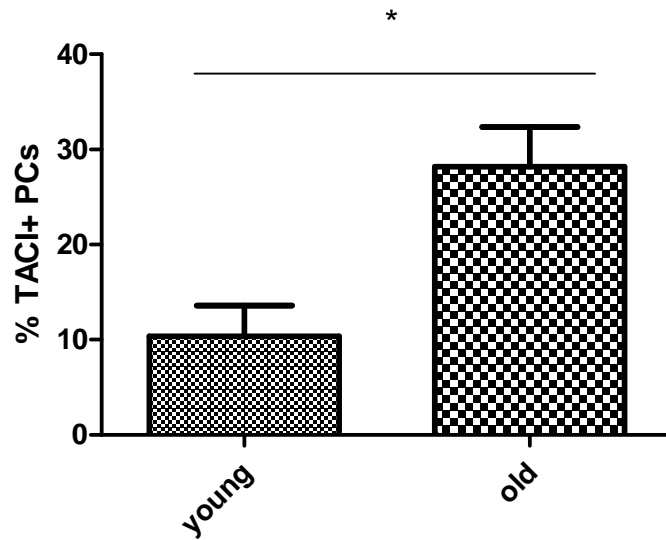


Figure 22: Comparison of TACI positive plasma cells in young and old murine femoral bone marrow. The bone marrow from young (5-8 week old) and old (18-24 months) was isolated and stained for the cell surface markers B220, CD138, and TACI. Based on size and granularity, plasma cells were classified based on their lack of B220 and presence of CD138. TACI positive cells were then analyzed, and frequency was determined. Unpaired t-test was used, and $P = 0.0087$ * with $N = 6$ for young and 4 for old.

Samples	Average MFI	Standard Deviation
8-10 wk femoral bone marrow PCs	722	105
18-21 month femoral bone marrow PCs	733	265

Table 3: Mean Fluorescence Intensity (MFI) of TACI on femoral plasma cells.

FACS analysis determined if TACI was upregulated on individual cells, based on the MFI. An increase in the MFI suggests that on a single cell, there is an increase in the number of receptors on the cell surface. However, the MFIs were similar; suggesting that if a plasma cell is positive for TACI, the expression of TACI is similar, regardless of the age of the mouse.

SUMMARY

Overall, this chapter focused on the TNF family ligand, APRIL, and its cognate receptor, TACI. Since APRIL's role in B cell survival has been previously established, I sought to determine if the amount of APRIL transcripts would change as the host ages, since the B cell compartment is known to change. I concluded that the concentration of APRIL transcripts is elevated in both the primary (bone marrow) and secondary (spleen) lymphoid tissue in the aged mouse. However, we do not currently know if this increase in transcripts correlated with an elevation of available APRIL protein in the microenvironment. Furthermore, I questioned whether the expression of TACI also changed with age. Previous research on TACI demonstrated that it facilitates the number and function of plasma cells in both the spleen and bone marrow. My research suggests that as the mouse matures, the number of B cells that express TACI increases. Specifically, the frequency of plasma cells expressing TACI is significantly increased in the aged murine bone marrow, compared to young (Figures 20 and 21). My research suggests that a mechanism for B cell survival utilized in the aged host is mediated by APRIL and TACI. With their uniform elevation in the aged host, it is possible that these two work in concert to provide adequate survival signals from the environment to the individual B cells. However, additional work is necessary to determine if these two

components are required for survival in the aged host, or if they are one of a few 'redundant' pathways for this process.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Understanding the humoral immune system is important in developing adequate methods to obtain appropriate protective immunity. Long-term plasma cells mediate this response while residing in the animal's bone marrow. This microenvironment promotes plasma cell maintenance and survival. The overarching goal of my thesis was to determine if the bone marrow is a static or dynamic environment in regard to the bone marrow-derived plasma and stromal cells and the signals required for humoral immunity. I addressed this issue in the murine model, using bone marrow location and age as my variables. Using both *ex vivo* (FACS, cytodots, and Real-Time PCR) and *in vitro* (co-cultures and ELISA) experiments, I addressed whether the femur and sternum bone marrows are equivalent (static) in regard to plasma cell frequency, survival, and APRIL expression. Coinciding with these experiments and additional ones examining the surface expression of APRIL's receptor TACI, I addressed the importance of age on the bone marrow microenvironment and wanted to conclude if age factored in the bone marrow's cellular composition, and plasma cell support. Based on the results, age contributes a prevalent role in the function, composition, and signal molecule availability in the bone marrow. The implications and future directions will be elaborated upon in the following sections.

CHARACTERIZATION OF PLASMA CELLS IN TWO DISTINCT BONE MARROW SITES

IgM production in femoral and sternum bone marrow. The aged bone marrow has been used recently as a source for plasma cells in unimmunized mice (62, 63). Though plasma cells are a rare cell population, with a frequency of roughly 0.5- 1% of the total aged bone marrow, their accumulation in the aged bone marrow allows for more efficient isolation for *in vitro* and *ex vivo* experiments. I first examined the plasma cell population via total bone marrow culture IgM production and cytodots. IgM is one of the five immunoglobulin isotypes, and is the primary immunoglobulin found in the circulation. This is largely due to IgM being the first immunoglobulin produced after an exposure to an antigen. These traits made IgM an ideal candidate in our naïve mouse model.

Assessing IgM concentrations in culture supernatants demonstrated an elevation in sternum bone marrow cells cultured at low cell densities (100,000 cells per well), but at higher cell densities, the two bone marrow sites had equivalent immunoglobulin concentrations. Interpretations for this finding include: (1) At lower femoral bone marrow cell concentrations, there are plasma cell factor(s) that are produced in limited amounts. For example, a lack of adequate signals, like APRIL, could affect the survival of these cells, which would affect the secretion of immunoglobulin. (2) At lower cell

densities, there may be a decrease in plasma cell niches, which facilitate their survival and function. At a higher density, these limited growth factors and/or niches could reach equivalent levels in the bone marrow from either location.

Alternatively, since plasma cell frequency was assessed indirectly in the previous experiment, both the femur and sternum may house equivalent numbers of plasma cells, despite the difference in immunoglobulin concentrations. ELISPOT experiments on isolated femoral plasma cells suggested that plasma cells are not uniform in immunoglobulin secretion since the spots are variable in size (Heather Minges Wols, unpublished data). Based on these observations, the equal concentrations of IgM found at 250,000 cells/well does not necessarily have to correlate with equivalent number of IgM producing plasma cells in the two bone marrow sites. The conclusions from this experiment are that these two sites appear equivalent in their production of IgM, which suggests that regardless of the location, the bone marrow microenvironment is uniform in protecting against an initial encounter with an antigen by producing equivalent amounts of IgM.

Assessment of plasma cells by cytodots. To specifically characterize the femur and sternum plasma cells, total bone marrow samples were examined by cytodots. Plasma cells that secreted either IgM, IgG or IgA were identified based on their morphology and fluorescence by assessment through microscopy. [Based on the criteria,

a ‘blinded’ additional investigator determined that an increased frequency of plasma cells was found in the aged femur and sternum bone marrow, when compared to young.] Additionally, in data not shown, when experiments were done combining the two marrows together, there was always an increase in the number of plasma cells in the aged compared to the young, demonstrating that in aged hosts, the marrow, as a whole, harbors an accumulation of plasma cells. Thus, the decline in overall humoral immunity in the elderly may not be due to an insufficient supply of plasma cells, but rather the amount and/or quality of the immunoglobulin they are producing. Since the fluorescence appeared similar in intensity between the young and old samples, I speculate that the decline is in support with previous findings that the quality of antibodies (overall affinity and diversity) negatively affects the protective humoral response in the elderly (30, 53, 80).

My results showed that the frequency of IgM, IgG, and IgA positive plasma cells in the sternum tended to be elevated compared to the femur in individual mice. However, this finding is not capable of distinguishing which type(s) of immunoglobulin producing plasma cells are different between the two bone marrow sites. Experiments assessing the intracellular immunoglobulin, individually, would help determine which plasma cell subset varied between the sternum and the femur. If the concentrations of IgM detected *in vitro* reflect equivalent number of IgM producers *in vivo*, then the experiments suggest

the sternum may be the host's prominent bone marrow site for IgA and IgG plasma cells. IgA is an antibody important for mucosal immunity. Speculatively, the sternum may be favored for the location of these cells since the sternum is in close proximity to the respiratory tract, which is one of the first sites to encounter and recognition of mucosal antigens. IgG is produced during the secondary infection or exposure to an antigen. Despite the fact that our mice were not challenged with known antigen, it is possible they were exposed to non-pathogenic antigens even in barrier colonies. However, without testing the presence of IgA and IgG positive plasma cells separately, we cannot conclude which population, out of the two, is truly different between the femur and sternum. These possibilities could be elucidated with additional FACS, cytodots, and co-culturing experiments, to assess the immunoglobulin production distinctly, instead of as a whole population. Moreover, experiments utilizing an infection model could determine if, *in vivo*, there is an elevation in the accumulation of these immunoglobulin isotypes in the sternum. Once each isotype is examined, speculation on *in vivo* significance can be determined and further investigated.

ARE THERE INTRINSIC DIFFERENCES IN THE *IN VITRO* SURVIVAL OF FEMORAL AND STERNUM PLASMA CELLS?

In vitro survival of plasma cells can be accounted for by the extent of IgM detected in culture supernatants. The viability of femoral and sternum plasma cells was

established when these plasma cells were co-cultured on stromal cells isolated from a single pool of long-term bone marrow cultures (Figure 9). At face value, the femoral plasma cells appeared to survive better than sternum plasma cells since their supernatants contained approximately 10 ng/ml more IgM per day. Despite the difference in concentration, both plasma cells demonstrated similar IgM/survival trends. Both the femoral and sternum plasma cells reached peak survival roughly one week after the initial co-culturing, and this peak was similar to results obtained by Dr. Heather Minges Wols. The peak concentration suggests that plasma cells started dying off after about one and a half weeks, but then the remaining plasma cells were able to maintain their survival during the second week. Massive cell death, as depicted by a five- fold decline in IgM from both populations, was observed on day twenty one until day twenty four when the experiment concluded.

Despite the similar trends, the question remains as to why sternum plasma cells produce less IgM when compared to femoral plasma cells from the same mouse. It is possible that sternum plasma cells naturally turn over more rapidly than ones found in the femur. Future experiments could examine apoptosis of plasma cells in our *in vitro* co-culturing system via ELISA and FACS. The interpretation that the plasma cells themselves are the cause for the decline in immunoglobulin could be misguided, since it has yet to be determined if femoral and sternum stromal cells have similar expression of

adhesion molecules and production of signaling molecules *in vivo* and *in vitro*. More interesting, it is unknown if all plasma cells require the same support in their survival and maintenance. Hopefully, future work will seek to profile stromal cells, possibly by microarray or FACS screen to determine if these cells are uniform or distinct in this capacity. Co-culturing experiments utilizing femoral feeder layers, sternum plasma cells, and the addition of exogenous *in vitro* femoral plasma cell survival factors, such as IL-6, would help elucidate the degree of survival similarity of these two plasma cell populations.

DOES THE AGE OF THE HOST AFFECT STROMAL CELLS AND THEIR ABILITY TO SUPPORT PLASMA CELL SURVIVAL *IN VITRO*?

Since the aged marrow harbors an accumulation of plasma cells, I wanted to ask if the aged marrow had an enhanced ability to promote *in vitro* plasma cell survival. I sorted stromal cells generated from long-term bone marrow cultures from 6 week and 15 month old mice and cultured them with plasma cells from aged femoral bone marrow. I chose femoral plasma cells for this experiment since they were shown to survive better under *in vitro* conditions, when cultured with aged stromal cells, compared to sternum plasma cells (Figure 9). When femoral plasma cells were cultured on young femur-derived stromal cells, survival was low and similar to survival when plasma cells were cultured alone (Figure 10). In contrast, stromal cells derived from the femoral bone

marrow of older mice yielded an increase in plasma cell survival, as assessed by the IgM concentrations detected in their supernatants. When stromal cells from the sternum were used however, no difference in the level of survival of femoral plasma cells on the two ages of stromal cells was observed (Figure 11).

These results suggest that age impacts the ability of femoral stromal cells to support survival *in vitro*, but does not appear to impact sternum stromal cells. It is necessary to repeat this particular co-culturing experiment to determine if this finding is consistent and significant, or if this was a one-time phenomenon. The overall low levels of IgM detected (approximately 2-5 ng/ml per day) leaves me hesitant, and could be due to low overall plasma cell survival or possibly human error. In addition, previous data in the lab utilized young femoral stromal cells in plasma cell co-culturing experiments (62). In those experiments, stromal cells sorted from long-term bone marrow cultures derived from young mice, were capable of supporting plasma cells better than media alone. The same protocols were used, however our young bone marrow was generated from five to six week old mice while Dr. Minges Wols' young mice ranged in age from six to twelve weeks. This difference could represent a window of time that is essential in the maturation of bone marrow-derived stromal cells and the overall microenvironment. To determine if that is indeed true, long-term bone marrow cultures could be generated from six week, eight week, ten week, and twelve week mice and stromal cells from these

cultures could be used to run parallel co-culture experiments to assess if there are any differences in survival. Despite the similar protocols, I typically cultured my initial long-term bone marrow cultures a week or two longer than previous experiments. Perhaps this led to the expansion of one type of stromal cell (reticular or fibroblastic) at the expense of the other, altering the results. Moreover, despite our endeavors to prevent contamination of the sort with dead cells, the extended processing could have begun necrosis and/or apoptosis in the cultures that became evident only after the stromal cells were sorted and placed in the 96-well plate. Regardless of the possibilities, future experiments should culture the long-term bone marrow cultures for only four weeks, to prevent these additional variables, which could potentially alter the results.

DOES THE BONE MARROW SITE AFFECT STROMAL CELLS AND THEIR ABILITY TO SUPPORT PLASMA CELL SURVIVAL?

The previous findings (Figure 9-11) hinted at the idea that the residence of stromal cells may influence their ability to support plasma cells. In order to directly address this issue, femoral plasma cells were cultured in the presence of stromal cells from the aged femur or sternum of an individual mouse. This experiment illustrated that when femoral plasma cells are cultured on stromal cells from aged hosts, it doesn't matter where the bone marrow-derived stromal cells were from (Figure 12). This finding suggests that while age may factor into the function of stromal cells, the location of their

residence does not. In this experiment, the survival trends and overall IgM concentrations were similar to those found in Figure 8; however, the feeder layer from sternum provided a peak survival later (at day twenty one) than the one from the femur (at day ten). If this is reproducible, it would suggest that the sternum stromal cells may provide plasma cell survival factors for a longer duration than the femur.

SUMMARY AND OVERARCHING HYPOTHESES GENERATED FROM

CHAPTER THREE

This chapter set out to determine if the microenvironments of the femur and the sternum are similar in ability to support plasma cells. The findings suggest that the bone marrow may be selective in the plasma cells they support. The sternum may harbor a higher frequency of IgM/A/G positive plasma cells than the femur. *I hypothesize that the sternum is primed to harbor plasma cells generated to produce antibodies (IgA and IgG) in response to secondary exposure of antigens.* Additionally, it is possible that intrinsic differences may be present within the plasma cells from these two locations. When the plasma cells from either site were co-cultured on the same feeder layer (aged femur-derived stromal cells), femoral plasma cells had better odds of surviving compared to sternum-derived plasma cells. *This result led me to hypothesize that the interaction between plasma cells and their niches is unique and based on their appropriate residence.* Last, this section demonstrated that the aged bone marrow-derived stromal cells increase the survival of *in vitro* cultures of plasma cells. *I hypothesize that stromal cells from aged mice facilitate the accumulation of plasma cells in the aged host.*

Overall, Chapter Three highlights the pitfalls of generalizing the interpretation from femoral bone marrow to represent all marrow in the animal. Additionally, this section may help spark the pursuit of mechanisms that regulate and maintain plasma cells

and their corresponding niches. Understanding these mechanisms and requirements can help aid the advancement of vaccines that will adequately protect our elderly population.

DOES THE EXPRESSION OF APRIL CHANGE IN AGED MICE?

The previous chapter strengthened previous findings in our lab demonstrating that aged microenvironment is different from the ones found in younger mice (41, 61, 62, 82). To delineate a potential mechanism that may be altered in the aged murine bone marrow, I examined APRIL and one of its receptors, TACI. This was based on published data supporting their importance in B cell function and survival (5, 7, 13, 19, 29, 38, 55, 56, 68, 74). Since the B cell subsets mature and differentiate in both the primary and secondary lymphoid tissues, I assessed both the bone marrow and spleen for changes in the concentration of APRIL transcripts. Regardless of the tissue examined, APRIL transcripts were elevated in the aged mouse, as determined by the ratio of APRIL to the housekeeping gene HPRT (Figures 146-1). These findings demonstrate that the microenvironment has potential to produce a varied concentration of important growth factors and signaling molecules, like APRIL.

Additional experiments are required to determine if the elevation in APRIL transcripts correlates with an increase in the secreted APRIL protein. Multiple approaches could be utilized to address this question. Quantative analysis, by ELISA, would verify the concentration of APRIL present in fresh bone marrow or secreted by total bone marrow and splenic cultures *in vitro*. *In situ* hybridization and/or immunofluorescence staining of bone marrow and splenic sections would help identify

APRIL producing cells and determine if production is localized to particular cells and/or locations. These approaches would allow for comparisons between young and old in regard to 'who' is producing APRIL in the various microenvironments. It is feasible to think that the composition of cells producing APRIL changes with age, in addition to the amount of APRIL being produced per cell. After the initial survey of the tissue for APRIL production, clues from those findings can be employed to assess specific cell types for intracellular APRIL by FACS.

APRIL expression between bone marrow sites. Previous findings suggest that the sternum bone marrow may harbor more IgM/A/G positive plasma cells, when compared to the femur (Figure 7). APRIL is important in isotype switching of immunoglobulins, suggesting that APRIL's function may be required more in the sternum than in the femur bone marrow (74). Real-Time PCR concluded that there was a slight elevation in APRIL transcripts in the sternum compared to the femoral bone marrow in individual mice (Figure 17). Although not statistically significant, the trend was similar, in comparison, between the plasma cell frequencies in the two bone marrow sites (Figure 7). Since isotype switching is a characteristic of secondary immune reactions, it would be of interest to assess the levels of APRIL in challenged mice. This would establish if an antigen challenge causes an increase in APRIL in the animal's bone marrow. Moreover, challenging mice with differently administrated antigens, such as

oral antigens, would help delineate the types of encounters (primary and/or secondary) that facilitate the expansion of available APRIL. Additionally, a challenged setting more closely mimics the *in vivo* condition of humans, allowing for more appropriate correlations to our physiological requirements.

Besides being a general growth factor for B cells, APRIL has been shown to specifically affect plasma cells. As previously stated, the number of plasma cells increases as the host gets older. One surface marker on plasma cells is the heparan sulfate proteoglycan, CD138 (Syndecan-1). Research has concluded that proteoglycans can bind to available endogenous APRIL in the host and *in vitro* and this interaction alone, or in concert with another APRIL receptor generates a signaling cascade in the cell (38, 74). The question remains as to whether the increases in CD138, in the aged animal, cause an up-regulation of APRIL, or vice versa. Adoptive transfer experiments could be used to answer this question. CD138 positive plasma cells, from aged murine bone marrow, could be transferred into the bone marrow of young mice. This would determine if the increase in CD138 correlates to an elevation of available APRIL. If the expression of CD138 leads to the production of APRIL, then young mice that contain the adoptively transferred plasma cells would have an increase in APRIL transcripts and protein, when compared to young mice without the transferred cells. Since plasma cells are difficult to

isolate and transfer, plasmablasts from the spleen of recently challenged aged mice could be used as well in this experiment.

The previous experiment would determine if the increased expression of CD138 and APRIL in the aged bone marrow correlate with one another. In order to delineate if the availability of APRIL to bind to CD138 regulates APRIL's production, old mice could be injected with a blocking antibody to CD138. By purposely blocking the receptor, this would conclude if the receptor's (CD138) availability to bind to its ligand regulates APRIL expression. If the availability to bind to CD138 affects APRIL expression, then in the mice injected with the blocking antibody, we expect to see a decline in APRIL transcripts by Real-Time PCR.

DOES THE EXPRESSION OF TACI ON B CELL SUBSETS CHANGE IN YOUNG AND OLD MURINE BONE MARROW?

Besides CD138, APRIL can also bind and signal through the TACI receptor on B cells. Whereas CD138 is restricted to plasma cells, TACI is expressed on multiple B lineage cells, including immature and mature B cells. FACS analysis was used to examine the expression of TACI on various B cell populations in the femoral bone marrow. The bone marrow from femurs was chosen for analysis since the B cell composition, unlike sternum bone marrow, has been examined in regard to changes with

age (Figure 3). Gated on lymphocytes, B cell lineage subsets identified based on B220 expression (Figures 18-20). These populations were then examined for relative TACI expression and the number of TACI positive B cells, per one-hundred thousand lymphocytes, was determined. The frequency of TACI positive mature B cells (B220 high) and plasma cells (CD138 positive, B220 negative) were significantly increased in the aged femoral bone marrow compared to the incidence found in the young femoral microenvironment (Figure 21).

Since the number of plasma cells normally increases in the aged murine bone marrow, I was not surprised to see a significant increase in TACI positive plasma cells in the previous experiment. To establish if the frequency of plasma cells expressing TACI was dependent on the animal's age, FACS was again used. TACI expression was assessed solely on the B220 negative, CD138 plasma cell population, and frequency was determined by dividing the number of TACI positive plasma cells by the total number of plasma cells found in the host's bone marrow. This approach revealed that the frequency of plasma cells expressing TACI was significantly increased in the aged femoral bone marrow, and that the previous findings were not based solely on the elevation in the plasma cell subset (Figure 22). Important to note is the fact that although this population increased, there was not an up-regulation of the expression of this receptor on the cell surface (Table 3).

Despite the fact that the frequency of TACI positive plasma cells increased, the majority of plasma cells were negative for the TACI receptor (Figure 22). If APRIL facilitates a critical function in the *in vivo* maintenance and function of plasma cells, then plasma cells must use additional APRIL receptors to receive this signal. Future research should look at the expression of BCMA, the final known receptor for APRIL, and determine if its expression also increases in the aged host. By assessing both TACI and BCMA on plasma cells would establish if the plasma cell only requires expression and signaling through one of these receptors for optimal *in vivo* preservation and function. FACS would determine surface expression of these receptors, and it would be interesting to see if there is a population that expresses both receptors. To conclude if the receptors are truly redundant, or if they facilitate unique properties, plasma cells could be isolated, based on their receptor expression, and sorted into *in vitro* stromal cell feeder layers. If the receptors are interchangeable, then I expect equivalent *in vitro* survival between TACI positive and BCMA positive plasma cells.

SUMMARY AND OVERARCHING HYPOTHESES GENERATED FROM
CHAPTER FOUR

This chapter set out to determine if the expression of the TNF family ligand, APRIL, and one of its receptors, TACI, changed in the aged femoral and splenic microenvironments. The data concluded that the aged femoral bone marrow and spleen generated more APRIL transcripts than the tissue of younger mice. *I hypothesize that the aged bone marrow and spleen generate more APRIL to aide in the function of plasma cells.* In particular, *I hypothesize that APRIL's increase correlates with the increased requirement for isotype switching.* Furthermore, the frequency of TACI positive plasma cells was elevated in the femur of aged mice. However, the majority of plasma cells were still negative for TACI. *I hypothesize that plasma cells need to express one other APRIL receptor, in addition to the proteoglycan CD138, to adequately respond to endogenous APRIL.*

Overall, Chapter four assessed the changes in the aged microenvironment, which is required to provide niches for various B cell subsets. Assessment of APRIL and TACI demonstrated that their expression increased in the murine primary and secondary lymphoid tissue. This change could facilitate the change in B cell composition that Dr. Kara Johnson identified in our lab nearly seven years ago. These findings brought up additional questions and highlighted potential mechanisms that can be investigated later.

APPENDIX

BM sample	APRIL copies (average)	HPRT copies (average)	APRIL:HPRT
8 week femur	147	8021	0.018
8 week sternum	177	7405	0.024
20 month femur	129	6730	0.019
20 month sternum	120	6218	0.021

Raw data to correlate with experiments for Figure 17

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